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(54) Title: HYALURONIC ACID AS DNA CARRIER FOR GENE THERAPY AND VEGF ANTISENSE DNA TO TREAT ABNORMAL RETINAL VASCULARIZATION

(57) Abstract

The invention provides methods and compositions for gene therapy, including antisense therapy. In one embodiment, the compositions comprise hyaluronic acid to promote uptake of nucleic acid by the target cells. The invention is illustrated with reference to treatment of retinal diseases caused by neovascularisation.

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HYALURONIC ACID AS DNA CARRIER FOR GENE THERAPY AND VEGF ANTISENSE DNA TO TREAT ABNORMAL RETINAL VASCULARIZATION

This invention relates to use of hyaluronic acid to target active agents which ablate the function of targeted genes in the control or treatment of disease. In one embodiment, this invention relates to a method and composition for treating ocular diseases, in particular retinal disease involving neovascularisation of the choroid and/or retina. It makes use of the phagocytic characteristic of specific cells in the eye to provide an effective manner of delivering an active agent to the target, for either short term or long term treatment of neovascularisation. The methods and compositions of the invention are useful for delivering DNA, RNA, anti-sense nucleotides, peptides or other therapeutic agents to phagocytic cells or surrounding cells.

BACKGROUND OF THE INVENTION

A) Hyaluronic Acid as an Adjuvant or Targeting Agent

Hyaluronic acid (HA) is a large, complex oligosaccharide consisting of up to 50 000 pairs of the basic disaccharide glucuronic acid-β(1-3) N-acetylglucosamine β(1-4). It is found *in vivo* as a major component of the extracellular matrix. Its tertiary structure is a random coil of about 50 nm in diameter.

HA has the ability to bind a large amount of water, which *in vivo* makes it a viscous hydrated gel with viscoelastic properties. It is found in this form in the mammalian eye, both in the vitreous and in the extracellular matrix.

HA has been used in the treatment of certain diseases and conditions of the human body both systemically and topically, because of its ability to target an active agent to sites where the disease or condition is localised (International Patent Publications No. WO 91/04058 and No. WO 93/16733). It has been shown that HA forms depots, for example at the injured carotid artery (relative to

uninjured contralateral arteries) and in colorectal tumours growing in experimental animals, and is retained in the skin of such animals. In all these cases, the sites of the deposits are areas of high HA receptor expression, indicating that HA targets specifically to tissues that are expressing high levels of these receptors, particularly to tissues undergoing unusual proliferation and migration, including tissues responding to injury, inflammation, development, and tumorigenesis.

The characteristic of HA which is important to its action as a potential adjuvant is its ability simultaneously to bind to other molecules and to bind to cell membranes. Cell surface receptors specific for HA have been identified, including the histocompatibility antigen CD44, the receptor for hyaluronic acid-mediated motility (RHAMM), intercellular adhesion factor (ICAM), and some homologous proteins in the CD44 family. The binding of virus to the cell membrane facilitated by HA would allow the usual endocytotic mechanisms of viral uptake to be more effective.

B) **Diseases of the Eye**

A variety of ocular diseases such as macular degeneration and diabetic retinopathy are characterised by neovascularisation of the choroid and/or retina. This process is the major cause of blindness in patients suffering from these conditions.

Prior Art Treatments

In age-related macular degeneration (ARMD), the formation and haemorrhaging of a subretinal neovascular membrane (SRNVM) results in rapid and substantial loss of central vision. Various treatments are available, but all are unreliable. Laser photocoagulation is the most acceptable type of treatment, but it still suffers from the disadvantages that damage by the laser rays causes dense, permanent scotoma (Schachet, 1994; Ibanez *et al*, 1995 and

Hudson *et al*, 1995) resulting in temporary loss of vision, and inability to prevent progression of the condition in the long term because of recurrence of the neovascular membrane.

Thus this treatment provides an advantage only in terms of preventing *profound* visual loss.

Similarly, surgical removal of the SRNVM or of subretinal blood, or re-positioning of the fovea by rotating the retina have largely been unsuccessful, due to post-surgical complications and to minimum or temporary improvement in vision. These invasive forms of treatment and the corresponding complications therefore far outweigh the advantages gained, and are limited in usefulness.

Administration of interferon α2a, which has some anti-angiogenic activity (Fung, 1991; Guyer *et al*, 1992 and Engler *et al*, 1994) and transplantation of retinal pigment epithelial (RPE) cells (Algvere *et al*, 1994) have also proved to be of limited usefulness, and initial promising results obtained with small groups of patients have not been confirmed in larger trials.

In addition to laser photocoagulation which, as described above, suffers from various disadvantages, the other main method of treating diabetic retinopathy is the control of blood glucose and blood pressure. The efficacy of such forms of treatment is limited by the motivation and compliance of the patient involved.

About 30% of the population above age 75 suffers from macular degeneration, and about 3 in 1000 individuals suffer from diabetic retinopathy. As each of these numbers will increase due to the aging of the population, and the increase in incidence of diabetes, there is a need for a more effective manner of treating these and other ocular diseases mediated by neovascularisation.

Mechanism of Neovascularization

Vascular endothelial cell growth factor (VEGF) is a dimeric, disulphide-bridged glycoprotein which is well-

known to be synthesised and secreted by a variety of normal as well as tumour cells. Recent observations indicate that VEGF is frequently detected in the neovascular retinal membranes of patients with diabetes (Malecaze *et al*, 1994), and in the ocular fluid from patients with either diabetic retinopathy or with central retinal vein occlusion (Aiello *et al*, 1994). More recently, it was found that VEGF expression was induced in conditions such as central vein occlusion, retinal detachment and intraocular tumours. In a rabbit model, levels of VEGF mRNA were elevated in the hypoxic region of the retina following induction of retinal vein occlusion. (Pe'er *et al*, 1995). Stimulation of VEGF expression by hypoxia has also been observed in other animal models (Pierce *et al*, 1995; Miller *et al*, 1994), and *in vitro* in all types of cell cultures (Simorre-Pinatel *et al*, 1994; Hata *et al*, 1995 and Thiema *et al*, 1995).

c) Anti-Sense DNA and Gene Therapy in Treatment of Diseases

The suppression of expression of genes encoding proteins which mediate undesirable activity has been achieved in a variety of situations by the introduction or *in situ* production of 'anti-sense' DNA sequences in the target cells. These anti-sense sequences are DNA sequences which, when transcribed, result in synthesis of RNA whose sequence is antiparallel to the sequence encoding the protein. Such anti-sense sequences have been tested in a number of viral diseases. Alternatively, anti-sense oligodeoxynucleotides can be introduced into target cells; such short sequences are not themselves transcribed, but inhibit transcription and/or subsequent translation of the corresponding sense DNA sequence in the target cell.

Until recently it was widely thought that the minimum sequence length necessary in order to effect anti-sense inhibition of gene expression was 12 to 14 nucleotides (Wagner, 1994). However, it has now been shown that the specificity of binding to the target

sequence can be sufficiently enhanced by use of modified oligonucleotides comprising C-5 propyne pyrimidines and phosphorothioate internucleotide linkages that sequences as short as 7 or 8 nucleotides are effective in providing gene-selective, mismatched sensitive, ribonuclease H-dependent inhibition, in which flanking sequences of the target RNA are important in determining specificity (Wagner *et al*, 1996).

However, successful use of anti-sense nucleotides to counter expression of a gene *in vivo* is limited by factors such as the need for specific suppression of mutant gene expression (Milan, 1993; McInnes and Bascom, 1992), or the need for high concentrations of the anti-sense nucleotides (Akhtar and Ivinson, 1993).

To date, this form of therapy has largely involved use of anti-sense sequences packaged in liposomes, or direct application of antisense cDNA or oligonucleotides to the site of disease. Thus attempts to increase uptake of anti-sense sequences into the target cell by encapsulating these sequences in liposomes have been largely unsuccessful. It is also difficult to target liposomes efficiently, and uptake is even lower than with viruses.

The targeting may also be achieved by virus-mediated DNA transfer, using viruses such as the Sendai virus. Sendai virus is an RNA virus which has been shown to deliver DNA and proteins into cells with more than 95% efficiency (Kaneda *et al*, 1987). In this gene transfer system, DNA nuclear protein complex in liposomes is directly introduced into the cytoplasm of the cell by the fusion activity of Sendai virus. The DNA is delivered rapidly into the nucleus with nuclear protein. Sendai virus-mediated gene transfer occurs by fusion of the virus with the cell membrane, and bypasses the endocytic pathway. Recently, highly efficient delivery of anti-sense or plasmid DNA into target cells by Sendai virus has been observed. Both the anti-sense and plasmid DNAs retained

their activity not only in culture but also *in vivo* (Kaneda *et al*, 1987). However, the use of this virus is limited by the fact that there are no suitable constructs available at present to use as vectors. In addition, the transferred DNA can only be expressed for a limited period of time since the gene transfer is mediated by fusion.

Retroviruses have been widely used for somatic tissue gene therapy (Boris-Lawrie and Temin, 1993). They can target and infect a wide variety of host cells with high efficiency, and the transgene DNA integrates into the host genome. Theoretically, the integration of the DNA will provide permanent production of the transgene which could result in permanent rescue of the cells. However, retroviruses cannot infect non-dividing cells (Salmons and Günzburg, 1993). Furthermore, the retrovirus particles are unstable *in vivo*, which makes it difficult to achieve high virus titre with inoculation. In addition, there are significant concerns regarding the oncogenicity of the integrated viruses. The inability of retroviruses to infect non-dividing cells means that they cannot be selected as candidates for gene transfer in the eye, as the most important target cells such as photoreceptors and RPE cells are non-dividing cells.

The usefulness of herpes simplex virus vectors has been limited by their poor efficiency of infection (Culver *et al*, 1992). Two types of vectors have been developed, namely the replication defective recombinants and the plasmid-derived amplicons. The latter requires a helper virus. Although the toxic genes can be removed from the herpes simplex virus with difficulty, the constructs remain cytotoxic (Johnson *et al*, 1992). In addition, the long term expression of the sequences inserted has been unsuccessful to date, and there are problems with the regulation and stability of the constructs. The application of modified herpes simplex viruses to the eye in gene therapy poses major concerns because of their pathogenicity. Herpes zoster virus infection causes

serious infections in the eye, frequently resulting in blindness requiring corneal transplantation.

Adenoviruses have been widely used for gene transfer in both non-dividing and proliferating cells. They can accommodate DNA up to 7.5 kb, and provide efficient transfection and high viral titre. The main advantage of using these rather than retroviruses is the ability to infect a wide range of non-dividing target cells (Kozarsky and Wilson, 1993). Replication-defective adenoviruses are considered to be relatively safe, in that these viruses are common pathogens in humans, usually causing relatively benign conditions such as colds. The vectors carry tumour genes with a deletion mutation, lowering the possibility of becoming oncogenic (Siegfried, 1993). In the first experimental gene therapy trial approved by the US National Institutes of Health Recombinant DNA Advisory Committee, recombinant adenoviruses were used to treat individuals suffering from cystic fibrosis.

However, the main disadvantage of adenoviruses is their transient gene expression. This is a result of the lack of integration of the transgene into the cellular genome. Furthermore, few attempts at gene delivery to non-dividing cells have been successful. The first successful gene transfer into the brain, which consists of non-dividing cells, was reported in 1993 using adenoviruses (Le Gal La Salle *et al*, 1993).

These results indicate that gene therapy is a theoretically viable approach in the treatment of diseases, but that the technical difficulties of efficient targeting and uptake need to be overcome by using viruses which adhere to and are taken up by the target cells. This process is inefficient, and the use of viruses may entail an undesirable level of risk of iatrogenic disease. Positive results have, however, been published that teach that regulation of biological processes by gene therapy is feasible.

There is therefore a need for improved methods of targeting gene therapy for the treatment of disease, and for suitable compositions comprising hyaluronic acid for use in such treatment.

D) **Gene Therapy and Ocular Disease**

In Australian Patent Application No. 75168/94 (Hybridon Inc), it was shown that *in vitro* expression of murine VEGF could be inhibited in COS-1 or NB41 cells by incubation with 19- to 21-mer anti-sense oligonucleotides based on murine VEGF. A 21-mer antisense nucleotide targeted against the translational stop site was shown to be the effective sequence. There is no disclosure or suggestion of specific targeting of sequences to any tissue in the eye, or of treatment of any ocular conditions other than diabetic retinopathy.

In U.S. Patent No. 5,324,654, a method of stimulating proliferation of non-malignant cells is disclosed. The method comprises the *in vitro* treatment of cells with an anti-sense nucleotide corresponding to the retinoblastoma (Rb) gene to inhibit expression of the Rb gene product, resulting in suppression of the expression of proteins which inhibit cell growth. In this way, proliferation of cells is encouraged. The proliferated cells can then be re-implanted if desired, and the cells may be genetically engineered to replace a specific gene prior to re-implantation. However, there is no reference to use of this anti-sense sequence to treat conditions of the eye. The invention of US-5324654 is directed to establishing cell lines capable of long-term proliferation and to treatment of conditions such as muscular dystrophy and diabetes, caused by failure to express a gene.

The targeting of a specific gene to a specific cell has not been attempted, and no one ocular type has been singled out. Specific targeting using adenovirus alone is expected to be difficult, as the virus has the ability to transfect a large variety of cell types.

For treatment of ocular diseases, in which other sites in the body are largely or entirely unaffected, it is highly desirable to deliver the therapeutic agent selectively to the target tissue in the eye. For anti-sense DNA, it is essential that the DNA be actually taken into these target cells.

The advances in gene therapy referred to above have led to further studies of the delivery and expression of transgenes into target cells, such as the β-galactosidase transgene into the retina (Bennett *et al*, 1994, Li *et al*, 1994 and Mashmour *et al*, 1994) using recombinant adenovirus as a delivery system. The retinal pigment epithelium (RPE) is a non-renewable single cell layer in the eye, situated between the neural retina and the choroid. The cells of the RPE are phagocytic neuroepithelial cells which form the outer most layer of the retina. The phagocytic properties of these cells have long been known, and have been reviewed (Bok and Young, 1979). High levels of transgene expression within 3 days in the RPE layer and within two weeks in the photoreceptor cells of the neural retina in young animals were observed. The expression of the reporter gene was followed up to 9 weeks. In older animals, neither subretinal nor intravitreal injections induced the expression of the β-galactosidase transgene in the photoreceptor cells (Li *et al*, 1994).

Australian Patent Application No. 61444/94 shows that replication-defective recombinant adenovirus is taken up by various tissues in the eye following injection into the anterior chamber, the vitreous humour, or the retrobulbar space, and that the reporter gene β-galactosidase is expressed. However, this document does not show that such forms of viruses successfully incorporate the active agent into the target cell or area. Nor is there any disclosure or suggestion that VEGF can be used to heal any ocular condition.

One specific obstacle to success of using anti-sense nucleotides as a form of therapy for the eye is the inability of the nucleotide to enter the target cells, and the limited stability of the oligonucleotides which have been modified, eg. phosphorothioate oligonucleotides (Helene 1991). These factors greatly restrict the success of gene therapy *in vivo*, particularly in the long term. In the treatment of retinal diseases, the ability to delay progression of the conditions by about 12 months would greatly increase the value and effectiveness of long term therapy.

Cytotoxicity has been observed in association with use of adenoviruses as a transport vector for retinal gene therapy. This cytotoxicity has been shown to be dose-dependent (Mashmour, 1994) and poses another difficulty in using such a vector. In order to decrease the dose of a given vector but retain its transfer efficiency, an adjuvant may be used. Adjuvants such as lipofectin have been shown to increase the uptake of "naked" DNA by cells.

Even though HA has been widely used in eye surgery as a replacement for vitreous humour lost during the surgical procedure, we are not aware of any suggestion in the art that HA promotes uptake of any pharmaceutical agent into any cells or tissues in the eye. Similarly, although HA has been suggested to promote penetration of pharmaceutical agents such as antibiotics or anti-cancer agents, as set out in Australian Patent Application No. 52274/93 by Norpharmco, this specification does not suggest that HA promotes uptake of any agent, let alone DNA or viruses, by *individual* cells of any type. In particular, this specification does not teach the use of HA via intra-ocular injection.

We have now found that the phagocytic nature of the RPE cells will increase the uptake of molecules such as oligonucleotides and viruses, following injection into the vitreous space *in vivo*. These RPE cells show increased uptake of virus compared to other cell types. Our findings

enable the induction of both long-term and short-term inhibition of VEGF expression in retinal or choroid epithelial cells, and hence inhibition of neovascularisation of the retina or the development of SRNVM.

SUMMARY OF THE INVENTION

According to one aspect, the invention provides a composition comprising a nucleic acid and a hyaluronic acid or a derivative thereof, together with a pharmaceutically-acceptable carrier.

The nucleic acid may be a DNA or RNA, and/or may be a nucleotide sequence which is in the anti-sense orientation to a target sequence. The target sequence is a nucleic acid sequence which is implicated in the causation or exacerbation of a pathological condition. This target nucleic acid sequence may be a genomic DNA, a cDNA, a messenger RNA or an oligonucleotide. Where the target nucleic acid sequence is a genomic DNA, it may be present in a coding region, or in a regulatory region, such as a promoter sequence.

Alternatively, the nucleic acid may be present in a vector comprising a nucleic acid sequence to be transferred into a target cell. Again the nucleic acid sequence may be genomic DNA, cDNA, messenger RNA, or an oligonucleotide. However, in this case the nucleic acid may either be a sense sequence to be provided to a target cell in order to exert a function, or may be an anti-sense sequence to be provided to inhibit the functioning of a nucleic acid present in the target cell.

The vector comprising the DNA to be transferred may be a virus, such as an adenovirus, an adeno-associated virus, a herpes virus or a retrovirus. The use of all of these classes of virus as vectors for gene therapy has been extensively canvassed in the art. Alternatively the vector may be a liposome.

The invention also provides a method of treatment of a pathological condition in a subject in need of such treatment, comprising the step of administering an effective dose of a composition according to the invention to said subject.

It will be clearly understood that the dose and route of administration will depend upon the condition to be treated, and the attending physician or veterinarian will readily be able to determine suitable doses and routes. It is contemplated that the compositions of the invention may be administered parenterally, for example by intravenous or subcutaneous injection, topically, for example adsorbed on gels or sponges, or directly into the tissue to be treated, for example by intra-ocular or intra-tumoral injection.

The subject to be treated may be a human, or may be an animal, particularly domestic or companion mammals such as cattle, horse, sheep, goats, cats and dogs.

In the compositions of the invention the nucleic acid or vector may simply be mixed with the hyaluronic acid, or may optionally be physically or chemically coupled to hyaluronic acid. Methods for attaching DNA to hyaluronic acid have been disclosed in "Synthesis of Sulfonated Hyaluronan Derivatives containing Nucleic Acid Bases, Chemistry Letters, 1994 2027-2030 and "Transport Performance of Nucleosides Through Nucleic Acid Bases Conjugated to Hyaluronan"; Chirachanchai, S., Wada, T., Inaki, Y. and Takemoto, K, Chemistry Letters. 1995 2 121-122.

In a preferred embodiment this aspect of the invention provides compositions and methods for treatment of a retinal disease mediated by abnormal vascularization, in which the nucleic acid is an anti-sense nucleic acid sequence corresponding to at least a part of the sequence encoding vascular endothelial growth factor (VEGF), and is administered together with a hyaluronic acid as described below.

Many forms of HA are suitable for use for the purposes of the invention. In particular, both low and high molecular weight forms of HA may be used. The only requirement is that the HA be of a degree of purity and sterility to be suitable for pharmaceutical use; preferably the HA is also pyrogen-free. High molecular weight preparation of HA may require dilution prior to use. In particular, commercially-available HA products suitable for use in the invention are those supplied by Hyal Pharmaceutical Corporation, Mississauga, which is a 2% solution of HA having a mean average molecular weight of about 225,000; sodium haluronate produced by Life Core™ Biomedical, Inc.; Pro Visc (Alcon Laboratories); and "HEALON" (Pharmacia AB, Üppsala). It will be clearly understood that for the purposes of this specification, the term derivatives of HA encompasses homologues, analogues, complexes, esters and fragments and sub-units of HA.

Derivatives of HA which may be used in the invention include pharmaceutically-acceptable salts thereof, or fragments or subunits of HA. The person skilled in the art will readily be able to determine whether a given preparation of HA, or a particular derivative, complex *etc.* of HA, is suitable for use in the invention.

According to a second aspect, the invention relates to a composition for treatment of a retinal disease mediated by abnormal vascularisation, comprising an anti-sense nucleic acid sequence corresponding to at least a part of the sequence encoding vascular endothelial growth factor (VEGF), and optionally further comprising one or more adjuvants such as hyaluronic acid or a dendrimer compound for increasing cellular uptake, together with a pharmaceutically acceptable carrier. The use of dendrimer compounds to transport genetic material into target cells is disclosed in International Patent Application No. WO 95/24221 by Dendritech Inc *et al.*

The VEGF is most preferably human retinal pigment epithelial (RPE) or choroidal endothelial VEGF.

In separate embodiments, this aspect of the invention is directed to treatment for such retinal disease in the short term (up to about two months), the long-term (up to about one year), and indefinitely (for the life of the patient). In the first embodiment, for short-term treatment the invention provides one or more anti-sense oligonucleotides having 100% complementarity to a corresponding region of the VEGF gene. The oligonucleotide should have 16 to 50 nucleotides, preferably 16 to 22, and more preferably 16 to 19 nucleotides. Modified oligonucleotides of the kind described by Wagner *et al* (1996) may be used, and enable the lower limit of sequence length to be reduced to 7 nucleotides.

For long-term inhibition, the invention provides a recombinant virus comprising VEGF DNA in the anti-sense direction. This VEGF DNA is a long sequence, which for the purposes of this specification is to be understood to represent a VEGF sequence of greater than 20 nucleotides in length, preferably greater than 50 nucleotides, ranging up to the full length sequence of VEGF. In this embodiment, the recombinant virus is accumulated in RPE cells, and produces anti-sense VEGF *in situ*, thereby inhibiting VEGF expression in the RPE cell.

For indefinite inhibition, the invention provides a virus comprising VEGF DNA in the anti-sense direction in which the virus is one capable of integrating the anti-sense sequence into the genome of the target cell. Preferably the virus is an adeno-associated or similar virus. As in the embodiment directed to long-term treatment, this VEGF DNA is of at least 20 nucleotides, preferably greater than 50 nucleotides. The adeno-associated or similar virus facilitates integration of anti-sense VEGF DNA into the RPE cell genome, thus enabling expression of anti-sense VEGF for as long as the cell remains functional.

Eye diseases which may be treated using the compositions and methods of the invention include, but are not limited to, age-related macular degeneration (ARMD) and diabetic retinopathy. Other ocular conditions and tissues in which neovascularisation occurs, for example branch or central retinal vein occlusion, retinopathy of prematurity (also known as retrolental fibroplasia), rubeosis iridis or corneal neovascularisation, may also be treated by the invention.

In another aspect, the invention provides a method of prevention or amelioration of a retinal disease mediated by abnormal neovascularisation, comprising the step of administering an effective amount of an anti-sense nucleic acid sequence directed against VEGF into the eye, thereby to inhibit neovascularisation.

The anti-sense sequence may be carried in a replication-defective recombinant virus, as a vector or vehicle. The vector preferably comprises replication-defective adenovirus carrying promoters such as the respiratory syncytial virus (RSV), cytomegalovirus (CMV), adenovirus major late protein (MLP), VA1 pol III or β-actin promoters. The vector may also comprise a polyadenylation signal sequence such as the SV40 signal sequence. In a particularly preferred embodiment, the vector is pAd.RSV, pAd.MLP, or pAd.VA1. In a more particularly preferred embodiment the vector is Ad.RSV.aVEGF or Ad.VA1.aVEGF.

In a preferred embodiment, human VEGF is subcloned into the vector, in order to create the restriction sites necessary for insertion, to form an adenovirus plasmid carrying VEGF or partial sequences thereof in an anti-sense direction, which can then be linearized by restriction enzyme digestion. The linearized plasmid can then be co-transfected with a linearized replication defective adenovirus, in a suitable permissive host cell such as the kidney 293 cell line.

The compositions of the invention may be delivered into the eye by intra-vitreal or sub-retinal

injection, preferably in an appropriate vehicle or carrier. Such methods of administration and vehicles or carriers for such injection are known in the art. Alternatively, *ex vivo* delivery of the compositions of the invention may be achieved by removal of RPE cells from the patient to be treated, culturing the cells and subjecting them to infection *in vitro* with a replication-defective adenovirus or an adeno-associated virus as defined above. RPE cells carrying the virus are then injected into the sub-retinal layer of the eye of the patient.

While the invention is specifically described with reference to conditions of the eye, the person skilled in the art will be aware that there are many other pathological conditions in which VEGF is of importance. Such a person will understand that the antisense oligonucleotides and the recombinant viruses of the invention are applicable to treatment of such other conditions. Similarly the skilled person will understand that while the invention is specifically illustrated with reference to VEGF the methods described herein are applicable to use with other proteins.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1a shows the results of GeneScan analysis of persistence of anti-sense oligonucleotides *in vivo* in the retina following a single intra-vitreal injection.

Figure 1b shows a confocal microscopic image of the retina of a RCS-rdy^{+} rat at different times following injection of CATSCF.

Figure 2 is a graphical representation of the number of phagosomes in the RPE layers of Long-Evans rats. Doses were as follows: Low 6.6 μg, medium 66 μg and high 132 μg of CATSC anti-sense oligonucleotide. Each column shows the mean and standard deviation of the number of phagosomes in five randomly selected areas in the rat retinas.

Figure 3 is a graphical representation of the number of phagosomes in the RPE layers of RCS-rdy+ rats. Experimental animals were injected with 66 μg of sense oligonucleotides (S1) and 66 μg of antisense oligonucleotide (CATSC).

Figure 4 shows the effect of increasing the titre of adenoviral vector on the number of cells expressing the adenoviral transgene. In all cases, the incubation period was 16 hours. RPE7 denotes Human retinal pigment epithelial cells from a 7 year old donor; F2000C denotes F2000 fibroblastic cells. The C suffix on the F2000 key indicates that the counts for the F2000 cell expression have been corrected for direct comparison with the RPE7 cells.

Figure 5 shows the effect of increasing the time of incubation with the adenoviral vector on the number of cells expressing the adenoviral transgene. In all cases, the concentration of the adenoviral vector was 2×10^6 p.f.u./ml. The C suffix on the F2000 key indicates that the counts for the F2000 cell expression have been corrected for direct comparison with the RPE7 cells.

Figure 6 is a graphical representation of the effect of Hyaluronic Acid (HA) on the number of RPE7 cells expressing an adenoviral transgene for a fixed viral titre. The three bars indicate the effect of 0.001% HA, 0.005% HA and no HA (control). The error bar indicates one standard deviation.

Figure 7 is a graphical representation of the effect of Hyaluronic Acid (HA) on the number of F2000 cells expressing an adenoviral transgene for a fixed viral titre. The three bars indicate the effect of 0.001%HA, 0.005%HA and no HA (control). The error bar indicates one standard deviation.

Figure 8 shows the immunofluorescent staining of HA receptors in RPE7 and F2000 fibroblasts 8a. CD44 staining on RPE7; 8b. ICAM staining on RPE7; 8c. RHAMM staining on RPE7; 8d. CD44 staining on F2000 fibroblasts;

8e. ICAM staining on F2000 fibroblasts; 8f. RHAMM staining on F2000 fibroblasts.

Figure 9 shows micrographs of choriocapillary endothelial cells isolated from porcine eye, illustrating their characteristic appearance (top panel), presence of Factor VIII-related antigen (middle panel), and ability to take up acetylated low-density lipoprotein into the cytoplasm (bottom panel).

Figure 10 shows the effects of a variety of hyaluronic acid preparations on tube formation by choriocapillary endothelial cells.

Figure 11 shows the alkaline phosphatase staining of CD44 antigen in retinal pigment epithelium cells. In each case the epithelium is at the bottom of the picture with choroid above.

A. Unbleached pigment epithelium layer

B. Pigment epithelium layer bleached to remove melanin granules.

C. Bleached pigment epithelium stained with alkaline phosphatase-labelled anti-CD44 antibody.

Figure 12 shows the results of DNA PCR and RT-PCR analysis of transfection of a retinal pigment epithelial cell line with $VEGF_{165}$.

Figure 13 shows the effect of $VEGF_{165}$ produced by transfected RPE cells on tube formation by choriocapillary endothelial cells.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described by way of reference only to the following non-limiting examples. In some of these examples, the feasibility of the methods utilised in the invention is demonstrated using anti-sense oligonucleotides complementary to cathepsin S (CATSC).

Example 1 **Accumulation of Antisense Oligonucleotides in the RPE Cell Layer**

Human retinal pigment epithelial cells were cultured and on the third passage were used for *in vitro* experiments. Confluent cultures were incubated with bovine rod outer segments (ROS) to mimic the *in vivo* situation. A fluorescein-labelled anti-sense oligonucleotide complementary to human cathepsin S (CATSCF) was added to the medium of these cells and after 7 days of incubation, the cells were harvested. The presence of fluorescein-labelled oligonucleotides within the RPE cells was detected by fluorocytometry (FACS). A GeneScan DNA analyser was used to assess the presence and stability of the oligonucleotides in the cells. The fluorescence of cultured RPE cells was increased by about 100-fold, demonstrating the presence of the anti-sense oligonucleotides within the RPE cells. These results are summarised in Table 1.

Table 1

Fluorocytometer measurements of human RPE cells incubated with or without complementary CATSCF

SAMPLE	FACS READINGS
RPE + ROS	5.94
RPE + ROS + CATSC	8.50
RPE + ROS + CATSCF	461.50

It was not known if the fluorescence was emitted by the full length CATSC or by degraded oligonucleotides. Using GeneScan, it was demonstrated that the fluorescence was largely due to a 19-mer oligonucleotide, which appeared at a position similar to that of CATSCF. Using a similar procedure, it was observed that CATSC oligonucleotides were still intact after 7 days of incubation.

Example 2 **Cellular Distribution of Oligonucleotides in Retinal Cells and Stability of Oligonucleotides Following Injection Into Eyes**

One nmole of CATSCF was injected into the vitreous humour of 6-week old non-pigmented RCS-rdy$^+$ rats, and the movement of the oligonucleotides were followed by confocal fluoromicroscopy. Fluorescein (1 nmole) was also injected as a control. Animals were euthanised 2 hours, 3 days and 7, 14 and 28 and 56 days after injection. Following euthanasia, the injected eyes were enucleated, frozen, sectioned and immediately used for confocal microscopy without fixation.

Two hours after intravitreal injection of CATSCF the penetration of the oligonucleotides were observed in the ganglion cell layer at 2 hours and also in the photoreceptor and pigment epithelial layers at 3 days. However, 7 days following injection, only the RPE layer had significant amounts of CATSCF. At 14, 28 and 56 days, a fluorescent signal was maintained in the RPE layer, and no signal was observed in any other cell types. These results show that a large proportion of CATSCF was taken up by the phagocytic RPE cells.

Following intravitreal injection as described above, eyes were dissected, the retina was removed, and the DNA extracted. The purified DNA was subjected to GeneScan analysis. The presence of undegraded fluorescein-labelled oligonucleotide was demonstrated in the rat retinas after 7, 14, 28 and 56 days of injection, as shown in Figure 1a. The intensity of the signal had significantly diminished by 56 days.

Confocal microscopic analysis was performed following a single injection of 10 nmol CATSCF into non-pigmented RCS-rdy$^+$ rats. Retinas were examined at intervals after injection, and the results are shown in Figure 1b, in which g represents the ganglion cell layer, i the inner nuclear layer, o the outer nuclear layer, and r

the retinal pigment epithelial layer. The panels show retinas 2 hours (B), 3 days (A), 7 days (C), 28 days (D) and 56 days (E) after injection of 10 nmol CATSCF, and 3 days (F) after injection of FITC as a control.

These results demonstrate that following intravitreal injection, oligonucleotides accumulate in the RPE cells. The oligonucleotides are present in the RPE layer up to 56 days, and remain in a biologically active form during this period of time.

<u>Example 3</u> <u>Biological Activity of Anti-Sense Oligonucleotides</u>

Female sixty day-old pigmented rats of the Long-Evans strain were obtained from Charles River Breeding Laboratories, Wilmington, MA.

Sixty day old non-pigmented RCS-rdy^{+} rats were obtained from our colony. The animals were acclimatised to a 12 hr light/ 12 hr dark lighting cycle, with an average illuminance of 5 lux for at least 10 days prior to experimentation.

Animals were anaesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Intravitreal injections through the pars plana were made using a 32 gauge needle. The left eyes served as controls, and the right eyes were injected with 3µl of 150 mM sodium chloride (saline), or with 3 µl of saline containing 6.6, 66 or 132 µg of CATSC respectively, an anti-sense oligonucleotide described earlier (Rakoczy *et al*, 1994) or 66 µg of sense oligonucleotide S1, 100% complementary to CATSC. Injected animals were allowed to recover from anaesthesia, and at one week post-injection were sacrificed by an overdose of sodium pentobarbital and used for morphological examination. All animals were killed within half an hour at the same time of the day, approximately 4 hours after light onset. Two to three animals were used for each dose.

Following enucleation, whole eyes were immersed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.125M sodium cacodylate buffer, pH 7.35. The cornea and lens were dissected free and the eyecup trimmed for orientation purposes. The tissue was fixed overnight at 4°C and then post-fixed for 1 hour in 1% osmium tetroxide at room temperature. After ethanol dehydration, the tissue was embedded in epoxy resin. Retinal sections were prepared for transmission electron microscopy as described previously (Kennedy *et al*, 1994).

Histological data were obtained by light microscopy. Semi-thin 1 μm sections were cut using a LKB 2088 Ultratome (LKB-Produkter, Sweden) with a diamond knife and stained with toluidine blue. The number of phagosomes that accumulated in the RPE cells of each specimen injected with saline, low (6.6 μg), medium (66 μg) or high 132 μg) dose of CATSC and 66 μg of S1 sense oligonucleotide was determined. From each eye, five sets of counts were made at 40 fold magnification and the standard deviation was calculated. Each set consisted of the total number of phagosomes in 250 μm length of RPE from 6 different randomly selected areas. The number of phagosomes that accumulated in the RPE of the control eyes, low medium and high doses of CATSC were analysed and graphically represented. Comparisons were made using the analysis of variance following the general linear models procedure of the SAS^R (version 6) statistical package (SAS Institute Inc., USA).

The results show that we successfully tested an anti-sense oligonucleotide (CATSC) in two strains of rats. The number of phagosome-like inclusion bodies present in control Long-Evans and RCS rdy + rats was not significantly different, 35.8+11.6 and 47.29+14.8 (mean ± SD), respectively, The intravitreal injection was non-traumatic. Light microscopic examination of the retinas of the saline injected eyes revealed no damage to the outer layers of the retina, and there was no increase in the

number of phagosome-like inclusion bodies in the RPE layer when compared to the control non-injected animals. Long-Evans rats were used to identify the minimum amount of CATSC required to induce biological changes in the RPE layer. In the control eyes and in those injected with low dose (6.6 μg) of CATSC, the number of phagosome-like inclusions within the RPE cells were 35.8+11.6 and 35.0+7.4 respectively. In animals injected with higher doses (66μg and 132 μg), the number of phagosome-like inclusions were 96.2+13.6 and 141.0+34.7, respectively, and the difference was statistically significant when compared to the control and low dose samples (Figure 2).

RCS-rdy+ rats injected with 66 μg of CATSC also demonstrated a statistically significant increase in the number of phagosome-like inclusion bodies, ie 204.20+39.3 when compared to the 47.20+14.8 in controls. In contrast, the injection of 66 μg of sense oligonucleotide (S1) did not increase the number of phagosomes (Figure 3) present in the RPE Layer, (34.4+12.54).

The inclusions found in RPEs of CATSC-injected Long-Evans and RCS-rdy+ animals were spherical in shape, and clearly distinguishable from the very dark, small elliptical melanin granules present in Long Evans rats. In the presence of 66 μg of CATSC, the tips of the outer segments showed signs of disorganisation and there were some vacuoles present in the outer nuclear layer. However these changes were not observed in S1 sense oligonucleotide-injected animals.

Electron microscopic examination of the RPE layer of a CAT SC-injected eye revealed no significant changes in the morphology of RPE cells. Melanin granules appeared smaller and less concentrated due to regional differences. Individual mitochondrial profiles were smaller in the treated group than in the controls, although the number was greater in the treated than in the untreated animals. Electron microscopic examination confirmed that the structures of the undigested material was similar to that

of phagosomes. The numerous phagosomes seen in the RPE layer of rats treated with CATSC were paranuclear, and contained mainly compacted phospholipid membranes, resembling undigested photoreceptor outer segment (POS) and confirming their photoreceptor origin. There were no other morphological changes observed in the POS layer, except for the disorganised appearance of the apices in treated animals.

<u>Example 4</u> <u>Gene Transfer to the RPE Cell Layer</u>

The nature and dynamics of gene transfer using an adenoviral vector were examined. The effects of adjuvants on the uptake of the adenovirus was also studied.

Human RPE cultures (HRPE7) were obtained from a 7-year old Caucasian donor and prepared as described in Rakoczy *et al* (1992). Human F2000 fibroblast cells were cultured, harvested and pooled in Minimal Eagles Medium (MEM, Multicel ™ Trace Biosciences, Australia), with 10% FBS (Multiser™, Trace Biosciences) and containing 125μl gentamicin (Delta West, Bentley, Australia) per 100 ml medium. One ml aliquots of the pooled cell suspension were placed into each well of a 24 well plate, to ensure equal seeding of wells. Experiments were carried out with cells at confluence, and at least two parallel sets of each experimental points were obtained.

Expression of Adenoviral Transgene

Replication-deficient Adenovirus 5 carrying a RSV promoter and β-Galactosidase gene (Ad.RSV.βgal) (Stratford-Perricaudet, 1992) was cultured and purified as described by Graham and Prevac, 1991. Ad.RSV.βgal was added to each well as a 1 ml aliquot, in MEM, at a concentration of $4x10^6$ p.f.u./ml. for the time-based trials, giving a final concentration of $2x10^6$ p.f.u./ml. For the titre-based trials, concentrations of $8x10^3$, $4x10^4$, $8x10^4$, $2.4x10^5$, $4x10^5$ p.f.u./ml were added to the wells in a 1 ml aliquot, making the total volume 2 ml in each well

(the final viral concentration is half of that added). All of the trials examining the effect of increasing viral titres involved incubation of the culture with the viral suspension for a fixed period of 16 hours.

Experiments were terminated by removing the medium from each well and fixing the cells with 0.5 ml of 0.5% glutaraldehyde. The glutaraldehyde was removed after 5 minutes and the cells washed once with Phosphate Buffered Saline (PBS). Following this, 0.5 ml of X-gal stain [For 1 ml of solution (concentration in final solution): 25µl X-Gal (0.5mg/ml, BioRad, Hercules, California), 44µl HEPES buffer (44mM), 100µl $K_4Fe(CN)_6$(3mM) 100µl $K_3Fe(CN)_6$(3mM, 100µl NaCl(15mM), 100µl $MgCl_2$(1.3mM), sterile distilled water to make 1 ml (531µl)] was added to each well and incubated overnight (about 16 hours) at room temperature.

Cell Counting

An Olympus TO41 phase contrast microscope (Olympus Optical Co Ltd, Tokyo, Japan) at a magnification of 200x was used. Counting was carried out by a single observer. A second observer then blind counted 25% of the samples as a countercheck. A counting graticule in the microscope was used to define the region for counting when averaging was used.

All cells staining positively with the X-Gal stain were counted. At low expression of transgene (< approximately 2000 cells/well), the entire plate was counted. When the cell count was higher, averaging was used. Cells were counted in five standardized regions and their average was used to calculate the total count for each well.

In the trials comparing the rate of expression in HRPE7 and F2000 fibroblasts, the figure for the number of F2000 cells expressing the gene was corrected. This correction reflects the different total cell number of each cell type in a confluent culture in a 24 well plate. The count for HRPE7 is $3x10^5$ per well and for F2000, it is

$2x10^5$ per well. The graphical figures (Figures 4 and 5) also contain corrected counts to allow direct comparison. Where there is no comparison between cell types, no alteration of the raw count is carried out.

In the titre-based trials, the profiles of expression were markedly different in terms of rate of increase and absolute expression. For HRPE7 cells, the expression rate appeared to have an exponential form, while in F2000 fibroblasts the profile was more linear. There was a widening gap in expression throughout the trial comparing titre. At higher viral titre, HRPE7 expression was an order of magnitude greater than F2000 cells. For the conditions and titres tried in this experiment there was an overall and constant increase in the number of cells expressing with increasing vector titre (Figure 4).

In the study of the effect of incubation time on the profile of transgene expression, the concentration of AdV.RSV.βgal was kept constant at $2x10^6$ p.f.u./ml. The profiles of expression of transgene in the two cell types were markedly different, both in terms of rate of increase and magnitude of number of cells expressing the gene. There was also a notable delay between the sharp increase in number of HRPE7 and F2000 fibroblasts expressing the gene. For HRPE7 cells, the upturn in expression rate occurred at 4 hours while in F2000 fibroblasts, it occurred at 24 hours. There is a "window" period between 4 and 24 hours where the HRPE7 expression is an order of magnitude greater than that of F2000 cells (Figure 5).

<u>Example 5</u> <u>Effect of HA as an Adjuvant on the Uptake and expression of the β-gal Gene using a Viral Vector</u>

HRPE7 and F2000 cells were aliquoted into 24 well plates. The cells were incubated as described in Example 4, and allowed to reach 95% of confluence. Solutions of 0.001% to 0.005% buffered sodium hyaluronate (HA) (1% Hyaluronic acid from rooster comb; HEALON,

Pharmacia AB, Uppsala, Sweden) were prepared with MEM. A dose of 10 µl of viral solution at a concentration of 4 x 10^6 p.f.u. was added to 10 ml of each of the diluted HA solutions and 10 ml of MEM for the control, and incubated for 30 minutes at 25°C with intermittent gentle shaking. To separate wells of the 24 well plate, 1 ml of each of the test and control solutions was added. There were four parallel samples for each test concentration and for the control, which were counted and averaged.

The viral/HA solutions were incubated with the cell cultures for 16 hours. Each experiment was terminated according to the procedure given in Example 4.

Table 2

Experiment 1: Expression in HRPE 7 Cells

	1	2	3	4	Mean
RPE 7/HA (0.001%)	17114	20776	18730	17998	19168
RPE7/HA (0.005%)	17688	22186	20258	22236	20592
RPE 7/Cont	10782	15480	16326	15266	14705

The mean number of HRPE7 cells expressing the transgene in each well for adenovirus alone was 14 705 (SD±2228). For adenovirus with 0.001% HA the mean number of expressing cells was 19 168 per well (SD 1561) and for 0.005% HA the mean was 20592 (SD 2143) (Figure 6). This shows an increase in number of cells expressing the transgene of 30.4% for 0.001% HA and of 40.0% with 0.005% HA.

As assessed by Student's t test, the probability of the significance of the increase in number of HRPE7 cells expressing the gene, when 0.005% HA is used, compared with the control, is 0.0097, which shows a level of significance of $p<0.01$. The significance reflects the

large difference between the means (20592 (test) v 14705 (control)) and the separation of the means by more than two standard deviations.

The t test probability of the significance of the increase in number of RPE7 cells expressing the gene, when 0.001% HA is used compared with the control, is 0.02931, which shows a level of significance of $p<0.05$. The reduced significance reflects the smaller difference between the means (19168 (test) v 14705 (control)).

Table 3

Experiment 2: Expression in F2000 Cells.

	1	2	3	4	Mean
F 2000/HA (0.001%)	4358	4620	4195	NA	4391
F 2000/HA (0.005%)	4506	3914	4759	4332	4378
F2000 Cont	3844	3652	3875	3748	3780

The protocols for examining the effect of HA on the expression of a transgene in F2000 fibroblasts were the same as that for HRPE7. The numbers of cells expressing transgenes were significantly less than for HRPE7, which is consistent with the results demonstrated in Example 4. The mean number of cells expressing in each well for adenovirus alone was 3780 (SD±100). For adenovirus with 0.001% HA, the mean number of expressing cells was 4391 per well (SD±214) and for 0.005% HA the mean was 4378 (SD355)(Fig. 7.). This shows an increase of 15.8% for 0.001% HA and of 15.5% with 0.005% HA in the number of cells expressing the adenoviral transgene.

The two-tailed Student's *t* test was used to assess the significance of the difference between the means for each set of experimental data. For each experiment, the means, the Standard error of the differences of the

means and the p value for the t test are given. In both experiments, HA gave very significantly increased uptake ($p < 0.05$).

The t test probability of the significance of the increase in number of cells expressing transgene for the F2000 fibroblasts with 0.005% HA, compared with the control, is 0.0044, which shows a level of significance of $p<0.01$. The high significance here reflects the large difference between the means (4391(test) v 3790(control)) and the small variation within the two samples. The standard deviation is 214(test) and 111(control).

The t test probability of the significance of the increase in number of cells expressing transgene for the F2000 fibroblasts with 0.001% HA, compared with the control, is 0.0195, which shows a level of significance of $p<0.05$. There is a greater variation in the raw figures, and the standard deviation is higher than for the 0.005% sample (355 v 214), which accounts for the higher p value.

Preliminary trials of chondroitin sulphate and lipofectamine as adjuvants were also carried out in order to assess the likely efficacy. These agents had no significant effect on gene expression in HRPE7 cells.

The following doses of adjuvants were also used:-

Table 4

HA Concentration

Amount of viral solution	0.05%	0.01%	0.005%	0.001%	Control	Control
5 µl	176[a]	318	319	316	279	282
10 µl	305[a]	906	802	645	623	609
25 µl	-[a]	714[b]	1682	1822	1478	1184
50 µl	-[a]	2772	2692	3328	2250	1822

The figures represent the effect of HA concentration on the uptake and expression of β-gal transgene. Increasing virus concentration resulted in an

increase in the number of β-gal expressing cells. The numbers represent the number of RPE cells staining positive for β-gal following 16 hours incubation of virus in the presence of HA in a 24 well plate (cc 2x10" pfu/ml).

[a] The viscosity of these solutions precluded adequate dispersion of the HA and made them very difficult to manipulate.

[b] It was not clear why this figure fell so far outside of the normal distribution of the other results.

<u>Example 6</u> <u>Effect of HA Molecular Weight on the Uptake and Expression of the βgal Gene Using a Viral Vector</u>

Adenovirus with a β-galactosidase marker gene and a RSV promoter (AdV.RSV.βgal) was cultured in cells of the K293 embryonic human kidney cell line. Supernatant was collected, and the concentration of virus was determined by serial dilution with 4 replicates of each dilution. The concentration of the virus was calculated to be 5×10^8 pfu/ml. The virus was suspended in MEM medium with 10% fetal bovine serum (FBS) and 125μl/100ml gentamicin.

Human Retinal Pigment Epithelial Cells (HRPE) were from a 20 year old donor and cultured in medium as described above. They were aliquoted into 24 well plates from the same stock and allowed to reach confluence. Fourth passage cells were used.

The following HA preparations were tested:

1. Hyal (MW approx. 300 000)
2. Provisc (MW approx. 1 900 000)
3. Healon GV (MW approx. 5 000 000)

Each of the preparations was diluted to a solution of 0.002% in MEM without FBS.

The virus solution as above was mixed in a 1:1 ratio with the adjuvant solution giving a final viral concentration of 2.5×10^8pfu and an HA concentration of 0.001%. The two solutions were incubated in this mixture

for 30 minutes at room temperature with gentle shaking. The control solution consisted of a mixture of the virus with MEM without FBS with no HA present.

To each of the 24 well cells 1 ml of the viral/HA mixture was added. Incubation was for 24 hours in a CO_2 incubator (5% CO_2) at 37°C. The experiment was terminated by removing the viral/HA mixture and adding 0.5ml of 0.5% glutaraldehyde for 5 minutes to each well. The well was washed once with PBS and reacted with X gal stain.

An Olympus TO41 phase contrast microscope (Olympus Optical Co. Ltd. Tokyo, Japan) at a magnification of 100X was used throughout. Counting was carried out by a single observer and checked against a second blind observer who counted a quarter of the samples. A counting graticule in the microscope was used to define the region for counting. All cells staining positively blue with the X-gal stain were counted as positive. Cells were counted in five standardized regions and their average was used to calculate the total count for each well. The results and statistical analysis are presented in Tables 5 to 9.

Table 5

(count is of sample only)	*Control*	*Hyal*	*Provisc*	*Healon GV*
Number of cells expression β-gal	2043	2486	2424	2756

Statistics

Anova: Single Factor Between all groups

Table 6

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	6129	2043	15769
Hyal	3	7458	2486	4225
Provisc	3	7271	2423.667	36677.33
Healon GV	3	8268	2756	36928

Table 7

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*
Between Group	777567.0	3	259189	11.07653
Within Groups	187198.7	8	23399.83	
Total	964765.7	11		

Anova: Single Factor Between Adjuvants

Table 8

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Hyal	3	7458	2486	4225
Provisc	3	7271	2423.667	36677.33
Healon GV	3	8268	2756	36928

Table 9

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	F_{crit}
Between Groups	187230.9	2	93615.44	3.61	0.094	5.14
Within Groups	155660.7	6	25943.44			
Total	342891.6	8				

There was an increase in transgene expression in all of the HA-containing samples relative to the control ($P < 0.003$). The percentage increase was 21.7%, 18.6% and 34.8% for Hyal, Provisc and Healon GV HA preparation respectively. There is no significant difference between the effect of different molecular weights of hyaluronic acid ($p = 0.09$).

These results demonstrate that hyaluronic acid increases viral vector uptake, demonstrating an adjuvant effect. In addition it was shown that the adjuvant effect is independent of the molecular weight of hyaluronic acid between MW 300 000-5, 000 000.

<u>Example 7</u> <u>Demonstration of HA Receptors on the cell membrane of HRPE7 and F2000.</u>

Polyclonal RHAMM (Receptor for Hyaluronan Mediated Motility) antibodies were kindly provided by Dr E Turley, Manitoba Institute of Cell Biology, Canada. The antibody was used at a dilution of 1:75. Monoclonal InterCellular Adhesion Molecule 1 (ICAM-1) antibodies (Boehringer-Mannheim) were used at a concentration of 4μg/ml and monoclonal homing receptor CD44 antibody (CD44) was used at a concentration of 4μg/ml (Boehringer Mannheim Biochemica, Germany). Monoclonal anti-human IgG antibody and rat non-immune serum were kindly provided by Dr M Baines, Lions Eye Institute, Perth, Australia. They were used at a concentration of 4μg/ml and a dilution of 1:75 respectively. Anti-Mouse IgG (Fab specific)-FITC conjugate secondary antibody was used at a 1:64 dilution and anti-Rabbit IgG (whole molecule)-FITC conjugate secondary antibody was used at a 1:100 dilution (Sigma Immunochemicals, St Louis, Missouri).

HRPE7 and F2000 fibroblast cells were cultured in Lab Tek 8-well slide chambers (Nunc Inc. Naperville, Illinois). Cell cultures were fixed with methanol at -20°C for 10 minutes before immunofluorescent staining. All primary antibody solutions were incubated for 1 hour. The primary antibodies used for each of the two cell types were monoclonal anti ICAM-1, anti-CD44 as test and monoclonal anti-Human IgG as control, and polyclonal anti-RHAMM with a non-immune rabbit serum as control. Following the removal of the primary antibody, each well was washed three times with PBS and the secondary antibody was applied for 1 hour. The secondary antibody to the monoclonal antibodies was

antimouse IgG and the polyclonal was anti-rabbit IgG. The secondary antibodies were applied to tissue without primary antibody as a further control. Finally, on removal of the secondary antibody, each well was washed a further three times before the well chambers were removed and the slides mounted with Immuno Fluore Mounting Medium (ICN Biomedicals Inc, Aurora, Ohio).

Immunohistochemical staining for CD44 using a monoclonal antibody demonstrated positive staining for both HRPE7 cells and F2000 fibroblasts, as shown in Figures 8a and 8b respectively. The staining had a distribution consistent with the cell surface, as the staining pattern was the same as the cellular outline of cultured tissue.

A monoclonal human anti-IgG was used as control, and was negative for both HRPE7 and F2000 fibroblasts. A second control, using secondary fluorescent antibody with no primary antibody was also negative for both cell types.

Immunohistochemical staining using a monoclonal antibody for ICAM-1 demonstrated positive staining for both HRPE7, and F2000 fibroblasts, as shown in Figures 8c and 8d respectively. The staining had a similar distribution to that of CD44, but the signal was slightly weaker. The same controls as for CD44 were used for ICAM-1 staining, and were also negative.

Staining for RHAMM receptors using a rabbit polyclonal antibody was positive for both HRPE7 and F2000 fibroblasts, as shown in Figures 8e and 8f respectively. The distribution of staining, however, was markedly different in the two cell types. In HRPE cells the staining pattern was predominantly nuclear, with a very faint cytoplasmic outline (Figure 8e). The distribution of staining in F2000 fibroblasts was similar to that of CD44 and ICAM-1, with no significant nuclear signal observable over the cytoplasmic or cell outline pattern.

The control serum was a rabbit non-immune serum, which was negative for HRPE7 but gave a very weak signal in F2000 fibroblasts. In both cases, the secondary

fluorescent antibody alone did not lead to a positive signal from either cell type.

Example 8 **The Effects of Hyaluronic Acid Preparations of Different Molecular Weight on Tube Formation**

Reagents

Hank's balanced salt solution (Hank's BSS) without calcium or magnesium, medium Hams F12, minimum essential medium with Earles salts (EMEM), foetal calf serum (FCS), penicillin-streptomycin, amphetericin B, and trypsin-EDTA were obtained from Australian Biosearch (Perth, Western Australia). Collagenase A, endothelial cell growth supplement (ECGF), mouse anti-human monoclonal antibody against factor VIII-related antigen, and anti-mouse Ig-fluorescein were acquired from Boehringer Mannheim Australia Pty. Ltd. (Perth, Western Australia). Gelatin, heparin, ascorbic acid were purchased from Sigma Chemical Company (Sydney, Australia), acetylated low-density lipoprotein (DiI-ac-LDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbo-cyanine perchlorate) from Biomedical Technologies, Inc. (Stoughton, Massachusetts), Matrigel from Collaborative Research (Bedford, Massachusetts), recombinant human vascular endothelial cell growth factor (VEGF) from Pepro Tech EC Ltd. (Rocky Hill, New Jersey), ProVisc (MW 1.9×10^6) from Alcon Laboratories, Healon (MW 2.5×10^6) and Healon GV (MW 5.0×10^6) from Pharmacia.

Isolation and Culture of Porcine Choriocapillary Endothelial Cells

Porcine eyes were obtained from a local abattoir 2-4 hours after death of the animals. The choriocapillary endothelial cells (CECs) were isolated as previously described (Morse *et al*, 1990, Sakomoto *et al*, 1995). Briefly, Hank's balanced salt solution (Hank's BSS) without calcium or magnesium, but with 0.1 % collagenase A was used to release endothelial cells at 37°C for 1 hour. After

washing twice in Hank's BSS, the cells were plated in 1 % gelatin-coated 75-cm^2 cell culture flasks in 5% CO_2, 95% air at 37°C. The growth medium consisted of Hams F12 plus 10% fetal calf serum (FCS), 100 U penicillin-100 μg streptomycin/ml, 2.5 μg/ml amphotericin B, 37.5 μg/ml endothelial cell growth supplement (ECGS), heparin 100 μg/ml, and ascorbic acid 25 μg/ml. After 24 or 48 hours of plating the capillary segments, the colonies of endothelial cells showing a cobblestone appearance flattened and spread. On the third or fourth day, the non-endothelial colonies were recognised and were circled with a permanent marker pen on the top of 75-cm^2 flasks. A glass pipette which had been drawn through a flame to produce a bead tip was used to remove and crush any non-endothelial colonies within the circles (Folkman *et al.*, 1979). This technique was carried out under a phase contrast microscope (x10 phase objective) in a laminar flow hood. The medium was changed twice to remove floating cells. This procedure was repeated three to five times to enrich the primary cells for endothelial cells before they became confluent. The cells were identified as vascular endothelial cells by typical cobblestone morphology, presence of factor VIII-related antigen (Sakomoto *et al*, 1995), and positive staining (uptake) with DiI-ac-LDL (Folkman *et al*, 1979).

The Effects of Hyaluronic Acid on Tube Formation

The tube formation assay was performed as previously described (Haralabopoulos, *et al*, 1994. Briefly, Matrigel (16.1 mg protein/ml) was prepared from the Engelbreth-Holm Swarm tumour was used to coat 24 well cluster plates (250 μl/well) as recommended by the product sheet. After polymerisation of the Matrigel at 37°C for 30 minutes in CO_2 incubator, 0.5 ml medium containing 10 or 20 μg/ml of hyaluronic acid preparations of different molecular weights (ProVisc, MW 1.9 x 10^6; Hyal MW 2.5 x 10^6 and HealonGV MW 5.0 x 10^6 respectively) in MEM with 10% FCS

was added to the Matrigel coated wells. 10% FCS in 0.5 ml MEM was used for comparison of a relative unit of the tube area. The CECs (passage 3-7) were lifted from flasks by 0.25% trypsin-0.02% EDTA, suspended in 5% MEM, and added to the coated wells (50,000 cells/well in 0.5 ml medium). To evaluate the areas of tube-like structures on the gel, photographs were taken with a phase-contrast microscope after six hours. Five to seven fields (x10 objective) were chosen randomly in each well for quantitative study.

Choriocapillary Endothelial Cells

Primary cultures of capillary endothelial cells have a characteristic appearance that distinguishes them from other cell types. In addition they were characterized by staining for factor VIII-related antigen, and assaying for the ability to phagocytize DiI-ac-LDL. More than 95% of the CECs showed a positive reaction to factor VIII-related antigen. Almost every cell showed uptake of DiI-ac-LDL into the cytoplasm, as shown in Figure 9. This indicates that at least 95% of the cells were choriocapillary endothelial cells (CEC cells).

Quantification of Tube Formation and Statistical Analysis

The tube areas from duplicate wells were measured using a Computer Imaging Analyzer System (Professional Image Processing for Windows, Matrox Inspector). The slide photographs were scanned into a computer and the background adjusted to obtain the best contrast between the tubes and Matrigel. Tube formation was then quantified by measuring the total tube area of each photograph. The results were expressed as the mean and the standard error of the percentage of tube area in the presence of 7.5% FCS alone (the final concentration) and were analyzed by Student's t-test for at least two experiments.

Tube Formation

After 1 hour of being seeded on the top of Matrigel, the CECs became attached. Within 2-3 hours the CECs rapidly migrated into a reticular network of aligned cells. After 3 hours the CECs started to flatten and form capillary-like structures on the surface of Matrigel. By 6 hours capillary-like structures became apparent, showing an anastomosing network like vessel tubes. Tube formation in control and experimental samples containing different preparations of hyaluronic acid at a biologically active concentration was assessed after six hours, and the results are summarized in Figure 10 and Tables 10 to 13.

Table 10

Pro VisK (5 µg/ml)	Pro Visk (10 µg/ml)	Healon (5 µg/ml)	Healon (10 µg/ml)
60.3	139.91	119.37	118.33
49.64	122.96	134.01	111.22
90.03	47.38	39.96	47.48
41.78	36.1	37.12	68.9
59.04	99.4	142.32	126.36
129.2	106.05	72.63	117.69

Table 11

Healon GV (5 µg/ml)	Healon GV (10 µg/ml)	Control
115.22	86.16	155.09
111.57	62.57	118.71
114.42	78.88	85.08
105.06	145.59	43.34
22.64	136.12	94.91
104.08		102.94

Table 12

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	6	429.99	71.67	1062.86
Column 2	6	551.8	91.97	1724.36
Column 3	6	545.41	90.90	2226.80
Column 4	6	589.98	98.33	1035.70
Column 5	6	572.99	95.50	1295.74
Column 6	5	509.32	101.86	1351.08
Column 7	6	600.07	100.01	1370.50

Table 13

ANOVA

Source of Variation	SS	df	MS	F	P-value	F_{crit}
Between Groups	3655.25	6	609.21	0.42	0.86	2.38
Within Groups	48984.10	34	1440.71			
Total	52639.35	40				

There was no statistically significant difference in CEC tube formation between the control and hyaluronic acid-containing samples, demonstrating that hyaluronic acid of molecular weight between MW 300,000-5,000,000 does not induce neovascularisation in the absence of another agent.

Example 9 **Demonstration of the Presence of CD44 HA Receptor in the Human Retina**

Preparation of Human Retina

A human Eye Bank donor eye was dissected following the removal of the cornea. After discarding the anterior segment the vitreous was carefully removed, leaving behind some parts of the neural retina and the complete layer of pigment epithelium attached to the choroid. The eye cap was filled with 2.5% glutaraldehyde for fixation. Sections of the fixed tissue were subjected to paraffin embedding. Paraffin blocks were cut and sections were transferred on to glass histochemical slides, dewaxed in xylane and ethanol, and washed in distilled water and Tris-buffered saline pH 7.2 (TBS).

Alkaline Phosphatase Staining of Sections

Removal of melanin granules was achieved by incubating the eye sections in 50 μl 0.25% potassium permanganate for 45 minutes followed by 50 μl 1.0% oxalic acid for 5 minutes. Bleaching was carried out following incubation with serum 50 μl/section of 10% normal horse serum/TBS (Commonwealth Serum Laboratories, Perth, Australia) for 30 minutes. Sections were then washed twice in TBS, 5 minutes per wash and incubated in 50 μl mouse anti-CD44 monoclonal antibody (Boehringer Mannheim Biochemica, Mannheim, Germany) or 50 μl of mouse anti-81-11 monoclonal antibody (non-immune control) for 60 minutes. After incubation sections were again washed twice in TBS, 5 minutes per wash, incubated in 50 μl of 1/250 horse anti-mouse IgG (H+L) conjugated to alkaline phosphatase conjugated to alkaline phosphatase (secondary antibody) for polyclonal antibodies for 60 minutes and washed twice in TBS, 5 minutes per wash. Sections were incubated in 50 μl FAST RED (Sigma Aldrich, St louis, USA) for 20 minutes, washed twice in TBS, 5 minutes per wash and counterstained in Meyer's Haemotoxylin for 10 minutes, followed by 5 minutes in tap water. Sections were allowed to dry, then

mounted using glycerol jelly.

Bleaching of melanin was carried out successfully without causing damage to the tissue sections, as shown in Figure 10. Staining of glutaraldehyde-fixed human eye sections with the mouse control monoclonal antibody and FAST RED resulted in clear staining of the RPE layer in unbleached tissue (Figure 10A) and following bleaching after incubation with 10% normal horse serum (Figure 10B). A strong pink signal demonstrating the specific presence of CD44 HA receptors in the retinal pigment epithelium but not in the choroid was observed in tissue stained with anti-CD44 monoclonal antibody (Figure 10C). As in choroid there was no signal detected in the neural retina.

These results demonstrate that the retinal pigment epithelial cells preferentially express HA receptors, thus facilitating an enhanced uptake of HA complexes.

<u>Example 10</u> <u>Up and Down Regulation of Cathepsin D Expression in NIH 3T3 Cells</u>

A 1620 bp HindIII fragment of human cathepsin D was subcloned into pHBApr-1-neo vector in both sense and anti-sense directions. Positive clones were selected, and the orientation of the fragments was confirmed by EcoRI restriction enzyme analysis. For the transfections of NIH 3T3 cells the clones carrying cathepsin D in the anti-sense and sense directions were on caesium chloride density gradients.

NIH 3T3 cells were seeded on to 6-well tissues culture plates at a concentration of 2 X 10^5 in 2 ml DMEM supplemented with 10% fetal bovine serum (FBS). The cells were incubated overnight at 37°C until they became 70% confluent. Having reached confluency, the cells were washed twice with serum and antibiotic-free medium. Lipofection reagent (10 µl) (GIBCO-BRL) diluted in 100 µl of OPTI-MEM (GIBCO-BRL) were gently mixed and incubated at room temperature for 15 minutes. Following incubation, an

additional 800 µl of OPTI-MEM was added to the mixture. This diluted mixture was gently overlaid onto the washed NIH 3T3 cells. The cells were incubated for 16-20 hrs before the transfection media was removed and replaced with DMEM supplemented with 10% FBS. After a further 48 hrs incubation the cells were trypsinised and subcultured at 1:5 in media containing 10% FBS and Geneticin 418 (GIBCO-BRL) at 1 ng/ml concentration. Successfully transfected cells selected with Geneticin 418 were maintained in media supplemented with FBS and Geneticin 418 as described above. Confluent transformed cultures were frozen for storage and subcultured for further analysis. The presence of cathepsin D in the transformed NIH 3T3 cells was detected with polyclonal antibody against cathepsin D, using a conventional cytochemical technique and an alkaline phosphatase-labelled second antibody.

The presence of cathepsin D fragment of the vector was demonstrated with HindIII digestion. Positive clones showed the presence of a 1620 kb fragment. The orientation was established by ECO RI restriction enzyme digestion, which gave two fragments at 5.7 and 5.9 kb in the case of the anti-sense orientation and 4.3 and 7.3 kb in the case of the sense orientation. All NIH 3T3 cells surviving Geneticin 418 selection carried cathepsin D clones, which are antibiotic resistant. The transformed control NIH 3T3 cells did not survive the selection procedure. The immunocytochemistry results suggest that NIH 3T3 cells carrying cathepsin D in the sense direction up-regulated cathepsin D production, while those carrying cathepsin D in the anti-sense direction down regulated cathepsin D production.

<u>Example 11</u> <u>Production of a $VEGF_{165}$-Expressing RPE Cell Line</u>

Cell Culture

The human RPE cell line 407A (Davis *et al*, 1995), was maintained at 37°C in a humidified environment

containing 5% CO_2. The culture medium consisted of Minimal Essential Medium (MEM, Trace Biosciences, Sydney, NSW, Australia) supplemented with 10% FCS (Trace Biosciences, Sydney, NSW, Australia) and 100 IU/ml Penicillin/100 μg/ml Streptomycin (P/S) (ICN Pharmaceuticals Inc, Costa Mesa, CA, USA). Cells were passaged 1 in 5 with 0.25% trypsin (Trace Biosciences, Sydney, NSW, Australia)/0.05% EDTA (BDH Chemicals Australia Pty Ltd, Kilsyth, VIC, Australia) approximately every 5 days.

Cloning of $VEGF_{165}$ into the Expression Vector

Mouse $VEGF_{165}$ in Bluescript KS was obtained from Dr Georg Breier, Max Planck Institut, Germany (Breier *et al*, 1992). $VEGF_{165}$ was inserted into the Bam HI site of pHβAPr-1-neo (Figure 1) (Gunning *et al*, 1987). This cloning was performed via pGem 7Zf(+) (Promega, Madison, WI, USA) for the addition of a Bam HI site to the 3' end of $VEGF_{165}$. A sense VEGF-pHβAPr-1-neo clone was identified by Eco RI digestion (Promega, Madison, WI, USA). VEGF-pHβAPr-1-neo DNA was prepared using the Qiagen Plasmid Midi Kit (Qiagen GmbH, Hilden, Germany). The extraction was carried out as described in the manufacturer's protocol, and the resulting pellet was resuspended in 500 μl TE buffer (10 mM Tris HCL, pH 8.0, 1 mM EDTA).

Transfection of RPE Cell Line

VEGF-pHβAPr-1-neo DNA was transfected into 407A cells using Lipofectin (Gibco BRL, Gaithersburg, MD, USA) as described in the manufacturer's instructions. Briefly, 2 mg of VEGF-pHβAPr-1-neo DNA in 100 μl OPTI-MEM (Gibco BRL, Gaithersburg, MD, USA) was mixed with 5 μl Lipofectin reagent in 100 μl OPTI-MEM. The mixture was allowed to stand at room temperature for 15 minutes, then made up to 1 ml with OPTI-MEM, and overlaid on to 60% confluent 407A cells. The cells were incubated at 37°C overnight in a humidified environment and 5% CO_2, then 4 ml MEM with 10% FCS and P/S was added. The cells were re-incubated for

24 hours before 1 mg/ml Geneticin (Gibco BRL, Gaithersburg, MD, USA) was included in the cell culture medium. After one week a series of discrete colonies was selected, and grown in 1 mg/ml Geneticin until established. The concentration of Geneticin was then decreased to 300 μg/ml cell culture medium.

A control cell line consisting of 407A cells transfected with pHβAPr-1-neo only (407A-pHβAPr-1-neo) was also produced using Lipofectin. Both cell lines were maintained in MEM containing 10% FCS, P/S and 300 μg/ml Geneticin.

Selection of Primers for DNA and RT PCR

Primers were selected to allow specific amplification of transfected mouse $VEGF_{165}$, without background amplification of human $VEGF_{165}$ from the human 407A cell line. The sequences of mouse $VEGF_{165}$ and human $VEGF_{165}$ as listed on the GenBank database were compared using the IBI Pustell Analysis Software (IBI Ltd, Cambridge, England). 19mer regions which were less than 70% homologous with human $VEGF_{165}$ were selected from mouse $VEGF_{165}$. Primer sequences were: "VEGFMO1", 115-134 bp on mouse $VEGF_{165}$, 5'-AGG AGA GCA GAA GTC CCA T; "VEGFMO2", 300-318 bp on mouse $VEGF_{165}$ 5'-CGT CAG AGA GCA ACA TCA C. Analysis of primer sequences by the Basic Local Alignment Search Tool (BLAST, National Centre for Biotechnology Information, Bethesda, MD, USA) demonstrated homology to mouse VEGF forms only.

DNA PCR

Cells were harvested using 0.25% trypsin/0.05% EDTA. Samples of 2 x 10^6 cells were collected and washed with PBS, then incubated for 3 hours, 37°C, in the presence of 100 ng/ml Proteinase K (Boehringer Mannheim, Mannheim, Germany) and 0.5% w/v Sodium Dodecyl Sulphate (SDS) (BDH Chemicals Australia Pty Ltd, Kilsyth, VIC, Australia). DNA was isolated by phenol/chloroform extraction and sodium

acetate/ethanol precipitation. DNA pellets were resuspended in 100 µl TE buffer.

All PCR reagents, including Ultra Pure Water, were obtained from Biotech International Ltd. (Bentley, WA, Australia). The PCR reaction mixture consisted of 5 µl 5X Polymerisation Buffer, 25 mM $MgCl_2$, 1U *Tth Plus* DNA Polymerase, 50 ng VEGFMO1, 50 ng VEGFMO2 and Ultra Pure Water to 25 µl. 1 µl of each DNA sample was used for PCR. For each series of PCR reactions carried out, a positive control containing 20 ng VEGF-pHβAPr-1-neo DNA, and a negative control containing Ultra Pure Water in the place of DNA, were included. PCR reactions were carried out using a Perkin Elmer GeneAmp PCR System 2400 Thermocycler (Perkin-Elmer Corporation, Norwalk, CT, USA). Cycles used were 1 cycle of 92°C for 5 minutes, 55°C for 1 minute, 74°C for 1 minute; 35 cycles of 92°C for 1 minute, 55°C for 1 minute, 74°C for 1 minute; 1 cycle of 92°C for 1 minute, 55°C for 1 minute, 74°C for 10 minutes. The PCR products were electrophoresed on a 2% agarose gel, and visualised by ethidium bromide staining.

Reverse Transcription PCR (RT PCR)

RNA was extracted using RNAzolB (Biotecx Laboratories Inc., Houston, Texas, USA). The procedure was carried out as described in the manufacturer's protocol, with RNA being extracted directly from confluent 25 cm^3 flasks of cells (4 x 10^6 cells per flask). The resulting pellets were resuspended in 50 µl Diethyl Pyrocarbonate (DEPC) (BDH Ltd, Poole, Dorset, England) treated water.

RT PCR was performed using the GeneAmp Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit (Perkin-Elmer Corporation, Norwalk, CT, USA). Reverse transcription and PCR reactions were carried out as described in the manufacturer's instructions. 200 ng RNA was used for each reaction. Water used for all reactions was Ultra Pure Water. The RT PCR positive control contained 20 ng of VEGF-pHβAPr-1-neo DNA. The negative

control received Ultra Pure Water in the place of RNA. Controls for DNA contamination were produced by the addition of *rTth* DNA Polymerase after completion of the Reverse Transcription step. RT PCR products were precipitated using sodium acetate/ethanol. Samples were washed in 70% ethanol and resuspended in TE buffer to 1/5 the PCR reaction volume. PCR products were electrophoresed on a 2% agarose gel and visualised by ethidium bromide staining.

Production of a $VEGF_{165}$-Expressing Retinal Pigment Epithelial Cell Line

$VEGF_{165}$ was successfully cloned into the Bam HI site of pHβAPr-1-neo. The identity of the clone was confirmed using a Bam HI digest which yielded two fragments of 10.0 kb, corresponding to pHβAPr-1-neo, and 656 bp, corresponding to mouse $VEGF_{165}$. Eco RI digestion of the VEGF-pHβApr-1-neo clone produced two fragments of 5.7 kb and 5.0 kb, confirming that VEGF was in the sense orientation.

VEGF-pHβApr-1-neo was transfected into the 407A cell line using Lipofectin. The presence of mouse $VEGF_{165}$ DNA in the transfected 407A cell line was confirmed using DNA PCR. DNA was extracted from VEGF-pHβApr-1-neo transfected 407A colonies, along with DNA from the control 407A-pHβApr-1-neo cell line. PCR of the VEGF-pHβApr-1-neo transfected 407A DNA resulted in the production of a 200 bp DNA fragment in every colony tested. This fragment was the size predicted from the position of the primers on the mouse $VEGF_{165}$ gene, and agreed with the fragment size produced from the VEGF-positive control. One established colony of transfected cells was chosen for the remainder of the experiments (407A-VEGF). No signal was detected on PCR of 407A-pHβApr-1-neo. The results are illustrated in Figure 11A. DNA from untransfected 407A cells also produced PCR signal, confirming that the primers being used were specific to mouse $VEGF_{165}$.

RT PCR was used to verify the production of mouse $VEGF_{165}$ mRNA by 407A-VEGF. On RT PCR of 407A-VEGF total RNA, a fragment of 200 bp was produced, corresponding to the fragment size predicted from the position of the mouse $VEGF_{165}$ primers. No signal was received from 407A-pHβApr-1-neo total RNA. Both RNA samples were shown to be free of contaminating DNA by omission of the cDNA synthesis step during RT PCR. The results are shown in Figure 11B. RT PCR using untransfected 407A RNA did not produce any signal.

Tube Formation Assay

The assay was performed as described in Example 8. CEC adhered to the Matrigel support within 1 hour of seeding. After 2 to 3 hours of culture, the CEC had migrated rapidly to form a reticular network of aligned cells, and subsequently began to form capillary-like structures on the surface of Matrigel. By 24 hours the CEC had the appearance of an anastomosing network, which is typical of vascular tubules. The quantitative analysis of tube formation, obtained from computer images, is summarised in Figure 13.

The most extensive capillary network was seen in CEC cultured in the presence of 100 ng/ml human recombinant VEGF (Figure 13B). The amount of capillary tube formation induced by the 407A-VEGF conditioned medium was similar to that from the human recombinant VEGF. In contrast the level of tube formation from conditioned medium of the control 407A-pHβAPr-1-neo cell line was significantly less, and was comparable to the control cultures containing Ham's F12 medium with 5% FCS and P/S only.

There was a 100% increase in the amount of tube formation induced by 407A-VEGF conditioned media when compared to 407A-pHβAPr-1-neo. This difference was found to be significant (P = 0.009, Student's t-test). The difference between the control culture and the culture containing 100ng/ml human recombinant VEGF was also found

to be significant (P = 0.002, Student's t-test).

Example 12 Cloning and Characterisation of Human RPE Vascular Endothelial Growth Factor (RPE-VEGF)

Human RPE cells, available in our laboratory, are grown in tissue culture. To upregulate VEGF expression, cell cultures are treated in hypoxic conditions. The upregulation of VEGF expression is monitored with immunohistochemistry. The mRNA is extracted from 10^7 RPE cells, and a cDNA library carrying all genes expressed in the RPE/choroid is established using methods known in the art.

VEGF is a highly conserved molecule which is highly homologous between different species. A murine VEGF cDNA clone, available in our laboratory, is used to screen the human RPE cDNA library in order to identify the full length human RPE-VEGF clone. Positive clones are analysed by restriction enzyme analysis and finally by DNA sequencing. Full length RPE-VEGF clones are analysed to elucidate their genomic structure (initiation sequences, start and stop codons, putative exons *etc.*).

Clones carrying the full length RPE-VEGF are analysed for the expression of VEGF protein with *in vitro* translation. The identified clones are used to derive the anti-sense molecule for insertion into the vehicle system, and for the selection of the anti-sense oligonucleotides.

Example 13 Pharmaceutical Agent for the Short-Term Inhibition of VEGF Expression

Anti-sense DNA technology enables the sequence-specific inhibition of target molecules without affecting the non-targeted functions of the cell. As described above, we have demonstrated both *in vitro* and *in vivo* that anti-sense DNA can be used effectively to inhibit the anti-sense oligonucleotide into the vitreous.

A panel of 16 to 19-mer oligonucleotides, 100% complementary to parts of the VEGF gene, is selected from the 5' and 3' ends of the DNA sequence. Sense and scrambled sequences are also used as control. Phosphorothioate-protected oligonucleotides are synthesized on a DNA synthesizer and subjected to purification.

Example 14 **Anti-Sense Agent for the Long-Term Inhibition of VEGF Production**

Preparation of VEGF-pAd.RSV for Homologous Recombination

$VEGF_{165}$ in Bluescript IIKS (Stratagene) was used to produce KpnI sites. Kpn I restriction enzyme sites were obtained at both the 5' and 3' ends of $VEGF_{165}$ by subcloning. $VEGF_{165}$ was removed from Bluescript II KS using an Xba I (5' cut)/Kpn I (3' cut) restriction enzyme digest, and cloned into pGem 7Zf(+) (Promega). A Kpn site was then added to the 3' end by cloning $VEGF_{165}$ into pGem 3Zf(+) (Promega), using a Hind III (3' cut)//Xba I (5' cut) digest.

VEGF was removed from pGem 3Zf(+) with a Kpn I restriction enzyme digest and cloned into the unique Kpn I site on pAd.RSV. This plasmid contains two segments of the adenovirus genome separated by cloning sites for the insertion of foreign DNA. The resulting clones were screened for the presence of sense and antisense clones, which were used in homologous recombination (VEGF-pAd.RSV). $VEGF_{165}$ was shown to be present and intact within pAd.RSV by restriction enzyme cleavage and sequencing.

VEGF-pAd.RSV DNA was prepared using the Qiagen Plasmid Midi Kit, as per the manufacturer's instructions. The DNA was linearised by Xmn I restriction enzyme digestion, purified by sodium acetate/ethanol precipitation and resuspended in TE buffer. The DNA was then stored at -20°C until required.

Generation of Ad.RSV-VEGF or Ad.RSV-aVEGF by Homologous Recombination

The adenovirus type 5 deletion mutant, d1324, was used to generate the recombinant adenovirus carrying VEGF. d1324 is unable to replicate due to deletion of the E1 region and, in addition, carries a partial deletion in the E3 region. In order to generate viral particles this mutant was propagated in 293 cells, which supply the missing E1 region in trans. The linearised plasmid DNA pAdRSV-VEGF or pAd.RSV-aVEGF was co-transfected into 293 cells with d1324 viral DNA which has had its extreme left-hand sequences removed by a Cla1 digestion. This reduces the infectivity of d1324 and allows for easier identification of recombinants. After transfection using the calcium phosphate precipitation method, screening of the resultant plaques yielded recombinant AdRSV-VEGF virus carrying VEGF in sense or antisense orientation.

<u>Example 15</u> <u>Construction of a Vehicle for the Permanent Expression of Target Molecules</u>

The vehicle described in Example 14 is suitable for long-term treatment in that it provides temporary (maximum one year) expression of the anti-sense VEGF DNA molecule, and consequent protection against neovascularisation. To achieve indefinite treatment, we use a vector system which enables the integration of VEGF in the anti-sense direction into the human genome present in RPE cells using an adeno-associated virus (AAV) vector, which means that the protection against neovascularisation can be provided for the rest of the life of the patient, as long as the RPE cells remain functional.

Adeno-associated viruses are non-pathogenic, are able to infect non-dividing cells, and have a high frequency of integration. We use AAV-2, which is a replication defective parvovirus which can readily infect other cells such as RPE cells, and integrate into the genome of the host cells. Recent characterisation has

revealed that AAV-2 specifically targets the long arm of human chromosome 19.

AAV constructs use varying promoter sequences in combination with a reporter gene. The expression of the reporter gene mRNA is detected with PCR amplification or *in situ* PCR, and the integration of the reporter gene is identified by chromosomal analysis of RPE cells.

Using the appropriate restriction sites, the reporter gene is replaced by anti-sense VEGF DNA. The new construct is co-transfected with the complementing plasmid (pAAV/ad) into kidney 293 cells previously infected with adenovirus type 5 to make the rAAVaVEGF construct. The construct produced is used to infect RPE cells, and the expression of anti-sense VEGF is detected by PCR amplification.

<u>Example 16</u> <u>Model Systems for Testing Inhibition *In Vitro*</u>

Human VEGF is cloned into COS cells to produce a culture system (VEGF-COS) in which the effective inhibition of VEGF expression can be tested. The inhibition of VEGF expression is tested by Northern and Western blot analyses and quantified by immunoassay.

The toxicity of increasing concentrations of oligonucleotides on COS cells is assessed with the trypan blue assay. The proliferation of COS cells is monitored with or without increasing concentrations of oligonucleotides. The inhibition of the expression of VEGF in controls and in cultures maintained in the presence of anti-sense oligonucleotides is monitored by Northern and Western blot analyses, immunocytochemistry and by a quantitative immunoassay.

RPE cells are cultured in hypoxic conditions and the up-regulation of VEGF expression is monitored in the presence of increasing concentrations of oligonucleotides for an extended period of time. Toxicity, proliferation assay and the monitoring of VEGF expression are performed

as described above.

CEC cells are cultured in normal and hypoxic conditions with or without increasing concentration of oligonucleotides. In addition to the toxicity, proliferation assay and VEGF detection, the effect of anti-sense oligonucleotide-mediated inhibition of VEGF expression on tube formation is analysed. RPE/CEC dual cultures produced in normal and hypoxic conditions will be subjected to similar tests. The same model systems are used to assess the long-term and permanent agents of the invention.

<u>Example 17</u> <u>*In Vivo* Model for Sub-Retinal Neovascular Membrane (SRNVM)</u>

In addition to the above examples an animal model for study of the particular inhibition of the development of SRNV was developed. The model uses laser treatment of rats to induce symptoms similar to those observed in humans as SRNV.

Pigmented rats (Dark Agouti, DA) weighing between 175 and 250 g were anaesthetized with an intramuscular injection of xylazine hydrochloride (2 mg/kg of body weight), acepromazine maleate (0.5 mg/kg), and ketamine hydrochloride (100 mg/kg of body weight) and given topical 0.5% proparacaine hydrochloride. The pupils were dilated with 2.5% phenylephrine hydrochloride.

Krypton laser radiation (647 nm) was delivered through a Zeiss slip lamp (Coherent Model 920 Photocoagulator, Palo Alto, Calif) with a handheld coverslip (22 c 30 mm) serving as a contact lens. Laser parameters used were as follows: a spot size of 100 µm, a power of 150 mW, and an exposure duration of 0.1 s. An attempt was made to break Bruch's membrane, as clinically evidenced by central bubble formation with or without intraretinal or choroidal hemorrhage. We found that a treatment power of 150 mW most consistently produced this effect.

Approximately 40% of animals treated the above described way developed growth of blood vessels into the retina from the choroid. This growth is accompanied by the upregulation of VEGF expression, providing an excellent system to test our oligonucleotides and constructs.

Example 18 Inhibition of RPE-VEGF Expression with Anti-Sense Oligonucleotides, Ad.RSV.aVEGF and rAAVaVEGF *In Vivo* in Rats

Neovascularisation can be induced using pocket implants in the choroid or the subretinal layer. One of the disadvantages of these models is that the process of neovascularisation might not follow the same biochemical steps which naturally occur in humans suffering from ARMD. To overcome these difficulties we use an animal model in which choroidal neovascularisation is induced by VEGF overexpression in the RPE cells. Using recombinant adenoviruses carrying VEGF, for example Ad.RSV.VEGF, for the *in vivo* trials all animal models described above are utilised to provide us with a wide range of information. Tests are conducted to demonstrate the expression of a VEGF expression over a period of one year. Using Northern and Western blot analysis, VEGF down-regulation is monitored and immunohistochemistry is used to demonstrate the down-regulation of VEGF expression in a cell-specific manner. Using the above described animal models, choroidal neovascularisation is monitored by histology and angiography. These models are applicable to all the embodiments of the invention.

It will be appreciated that the present invention is particularly useful in the study, treatment or prevention of age-related macular degeneration, by virtue of the successful adenoviral gene transfer to the RPE. Without wishing to be bound by any proposed mechanism for the observed advantages, the higher degree of gene expression in the HRPE7 cells, compared with the F2000

cells, may indicate the ability of RPE cells to phagocytose large molecules and hence increase the uptake of adenovirus. The level of expression of the transgene may also be increased by increasing the time of exposure or the viral titre.

The comparison studies between the HRPE7 cells and the F2000 fibroblast show that there are marked differences in the pattern of expression between the different cell types under the same conditions. These differences could be exploited for targeting of different cells, for example RPE. The upstroke in the time/expression curve for RPE cells (Figure 5) was at 4 hours, while for F2000 cells it was 24 hours. There is, therefore, a window during which RPE cells are taking up Ad.RSV.βgal. and expressing the transgene at a significantly higher level than F2000 fibroblasts. Transfection for periods of less than 24 hours would allow use of this window as a targeting tool (eg. virus solutions could be aspirated from subretinal blebs or the vitreous after 24 hours). The titre/expression curves (Figure 5) also show that there was a difference between the cells, with RPE cells beginning to express highly at a lower concentrations. Once again, low concentration could be used to preferentially target RPE cells. A combination of lower titres for less than 24 hours would combine the two effects and provide targeted delivery.

As shown in some of the embodiments, the present invention may also be used in conjunction with adjuvants to keep viral toxicity to a minimum by reducing the titre required to effect gene transfer and expression.

We have shown a consistent and significant adjuvant effect for adenoviral gene transfer using HA. This was the case in both phagocytic and non-phagocytic cell lines. The advantage of HA is its presence as a normal component of human vitreous and extracellular matrix, and its long history of therapeutic acceptance as a viscoelastic aid to surgery.

The important feature of HA in terms of its acting as a potential adjuvant is its ability to bind cell membranes and other molecules simultaneously. We propose that the HA molecule can bind adenovirus and the cell membrane at the same time, and therefore increase the contact time or concentration of virus in the vicinity of the cell membrane using this mechanism. We have identified cell surface receptors specific to HA identified on both F2000 and RPE7, as each cell tested positive for the presence of CD44, RHAMM and ICAM-1 receptors. Interestingly, the RHAMM receptors on RPE showed a nuclear distribution, and this could account for the slightly higher adjuvant effect of HA in RPE than in F2000. Our preliminary studies of *in vivo* immunofluorescent staining for CD44 show no signal in the neuroretina, suggesting that HA association of the adenovirus may also be a potential targeting mechanism for RPE *in vivo*.

It will be apparent to the person skilled in the art that while the invention has been described in the Examples, various modifications and alterations to the embodiments described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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CLAIMS

1. A composition comprising a nucleic acid and a hyaluronic acid or a derivative thereof, together with a pharmaceutically-acceptable carrier.

2. A composition according to Claim 1, in which the nucleic acid is a nucleotide sequence which is in the anti-sense orientation to a target sequence.

3. A composition according to Claim 2, in which the target nucleic acid sequence is a genomic DNA, a cDNA, a messenger RNA or an oligonucleotide.

4. A composition according to Claim 1, in which the nucleic acid is present in a vector comprising a nucleic acid sequence to be transferred into a target cell.

5. A composition according to Claim 4, in which the nucleic acid sequence to be transferred is a genomic DNA, a cDNA, a messenger RNA or an oligonucleotide.

6. A composition according to Claim 5, wherein the vector comprises a sense sequence to be provided to a target cell in order to exert a function.

7. A composition according to Claim 6, in which the vector comprises an anti-sense sequence to be provided to a target cell in order to inhibit the functioning of a nucleic acid present in the target cell.

8. A composition according to any one of Claims 1 to 7, in which the vector is a liposome.

9. A composition according to any one of Claims 1 to 8, in which the vector is a virus.

10. A composition according to any one of Claims 1 to 9, in which the virus is an adenovirus, an adeno-associated virus, a herpes virus or a retrovirus.

11. A composition according to Claim 9, in which the virus is a replication-defective adenovirus.

12. A composition according to Claim 11, where the virus is a replication-defective adenovirus comprising a promoter selected from the group consisting of respiratory syncytial virus promoter, cytomegalovirus promoter,

adenovirus major late protein (MLP), VA1 pol III and β-actin promoters.

13. A composition according to Claim 11, wherein the vector is pAd.RSV, pAd.MLP or pAd.VA1.

14. A composition according o Claim 11, wherein the vector is Ad.RSV.aVEGF or Ad.VA1.aVEGF.

15. A composition according to any one of Claims 10 to 14, wherein the vector also comprises a polyandenylation signal sequence.

16. A composition according to Claim 15, wherein the polyadenylation signal sequence is the SV40 signal sequence.

17. A method of treatment of a pathological condition in a subject in need of such treatment, comprising the step of administering an effective dose of a composition according to any one of Claims 1 to 16 to said subject.

18. A method according to Claim 17, in which the composition is administered systemically by injection.

19. A method according to Claim 17, in which the composition is administered topically.

20. A method according to Claim 17, in which the composition is administered directly into the tissue to be treated.

21. A method of preparing a composition according to any one of Claims 1 to 16, comprising the step of binding a nucleic acid or vector to a hyaluronic acid or a derivative thereof, and isolating the thus-formed complex.

22. A composition for treatment of a retinal disease mediated by abnormal vascularization comprising

a) an anti-sense nucleic acid sequence directed against vascular endothelial growth factor (VEGF), and

b) hyaluronic acid,

together with a pharmaceutically-acceptable carrier.

23. A composition according to Claim 22, in which the anti-sense nucleic acid sequence is present in a vector

comprising a nucleic acid sequence to be transferred into a target cell.

24. A composition according to Claim 23, in which the vector is a virus.

25. A composition according to Claim 24, in which the virus is an adenovirus, an adeno-associated virus, a herpes virus or a retrovirus.

26. A composition according to Claim 24 or Claim 25, in which the viral vector is a replication-defective recombinant virus.

27. A composition according to Claim 26, where the virus is a replication-defective adenovirus comprising a promoter selected from the group consisting of respiratory syncytial virus promoter, cytomegalovirus promoter, adenovirus major late protein (MLP), VA1 pol III and β-actin promoters.

28. A composition according to Claim 27, wherein the vector is pAd.RSV, pAd.MLP or pAd.VA1.

29. A composition according to Claim 27, wherein the vector is Ad.RSV.αVEGF or Ad.VA1.αVEGF.

30. A composition according to any one of Claims 1 to 29, wherein the vector also comprises a polyadenylation signal sequence.

31. A composition according to Claim 30, wherein the polyadenylation signal sequence is the SV40 signal sequence.

32. A composition for treatment of a retinal disease mediated by abnormal vascularization, comprising an anti-sense nucleic acid sequence corresponding to at least a part of the sequence encoding VEGF, and optionally further comprising one or more adjuvants for increasing cellular uptake, together with a pharmaceutically-acceptable carrier.

33. A composition according to Claim 32, wherein the anti-sense sequence has 100% complementarity to a corresponding region of the gene encoding VEGF.

34. A composition for short-term treatment according to Claim 32 or Claim 33, wherein the anti-sense sequence is 16 to 50 nucleotides long.

35. A composition for short-term treatment according to Claim 34, wherein the anti-sense sequence is 16 to 22 nucleotides long.

36. A composition for short-term treatment according to Claim 35, wherein the anti-sense sequence is 16 to 19 nucleotides long.

37. A composition according to Claim 33, wherein a modified oligonucleotide as herein defined is used, and the anti-sense sequence is 7 to 50 nucleotides long.

38. A composition according to any one of Claims 32 to 37 wherein the adjuvant is hyaluronic acid or a derivative thereof.

39. A composition for long-term treatment of a retinal disease mediated by abnormal vascularization, comprising a recombinant virus comprising an anti-sense nucleic acid sequence corresponding to at least part of the sequence encoding VEGF, together with a pharmaceutically-acceptable carrier, wherein the anti-sense sequence is between 20 nucleotides in length and the full length sequence encoding VEGF.

40. A composition according to Claim 39, wherein the anti-sense sequence is between 50 nucleotides long and the full length sequence of VEGF.

41. A composition according to any one of Claims 1 to 40, wherein the VEGF sequence is that of VEGF from human retinal pigment epithelial cells or choroidal endothelial cells.

42. A composition for treatment of a retinal disease mediated by abnormal vascularization, wherein said treatment is effective for an indefinite period, comprising a virus comprising an anti-sense DNA corresponding to at least part of the sequence encoding VEGF, together with a pharmaceutically-acceptable carrier, wherein said virus is one capable of integrating the anti-sense sequence into the

genome of the target cell.

43. A composition according to Claim 42, wherein the virus is an adeno-associated virus.

44. A composition according to Claim 42 or Claim 43, wherein the anti-sense sequence is between 20 nucleotides long and the full length sequence of VEGF.

45. A composition according to Claim 44, wherein the anti-sense sequence is between 50 nucleotides long and the full length sequence of VEGF.

46. A method of treatment of a retinal disease mediated by abnormal neovascularization, comprising the step of administering an effective amount of an anti-sense nucleic acid sequence corresponding to at least part of the sequence encoding VEGF into the eye(s) of a subject in need of such treatment, thereby to inhibit neovascularization.

47. A method according to Claim 46, wherein the anti-sense sequence is 16 to 50 nucleotides long.

48. A method according to Claim 46, wherein the anti-sense sequence is 16 to 22 nucleotides long.

49. A method according to Claim 46, wherein the anti-sense sequence is 16 to 19 nucleotides long.

50. A method according to Claim 46, wherein a modified oligonucleotide as herein defined is used, and the anti-sense sequence is 7 to 50 nucleotides long.

51. A method of treatment of a retinal disease mediated by abnormal neovascularization, comprising the step of administering an effective amount of a composition according to any one of Claims 22 to 45 to a subject in need of such treatment.

52. A method of treatment of a retinal disease mediated by abnormal neovascularization, comprising the step of administering a composition according to any one of Claims 39 to 41 to the eye(s) of a subject in need of such treatment, thereby to inhibit neovascularization in the long term.

53. A method of treatment of a retinal disease mediated by abnormal neovascularization, comprising the

step of administering an effective amount of a composition according to Claims 42 to 45 into the eye(s) of a subject in need of such treatment, thereby to inhibit neovascularization for an indefinite period.

54. A method according to any one of Claims 46 to 53, wherein the retinal disease is selected from the group consisting of age-related macular degeneration, diabetic retinopathy, branch or central retinal vein occlusion, retinopathy of prematurity, rubeosis iridis and corneal neovascularization.

55. A method of promoting uptake of an exogenous nucleic acid sequence by a target cell, comprising the step of exposing the cell to the nucleic acid, or to a virus or vector comprising the nucleic acid, in the presence of a hyaluronic acid or a derivative thereof.

56. A method according to Claim 55, in which the target cell is a phagocytic cell.

57. A method according to Claim 55 or Claim 56, in which the nucleic acid and hyaluronic acid are administered together *in vitro*.

58. A method according to Claim 55 or Claim 56, in which the nucleic acid and hyaluronic acid are administered together *in vivo*.

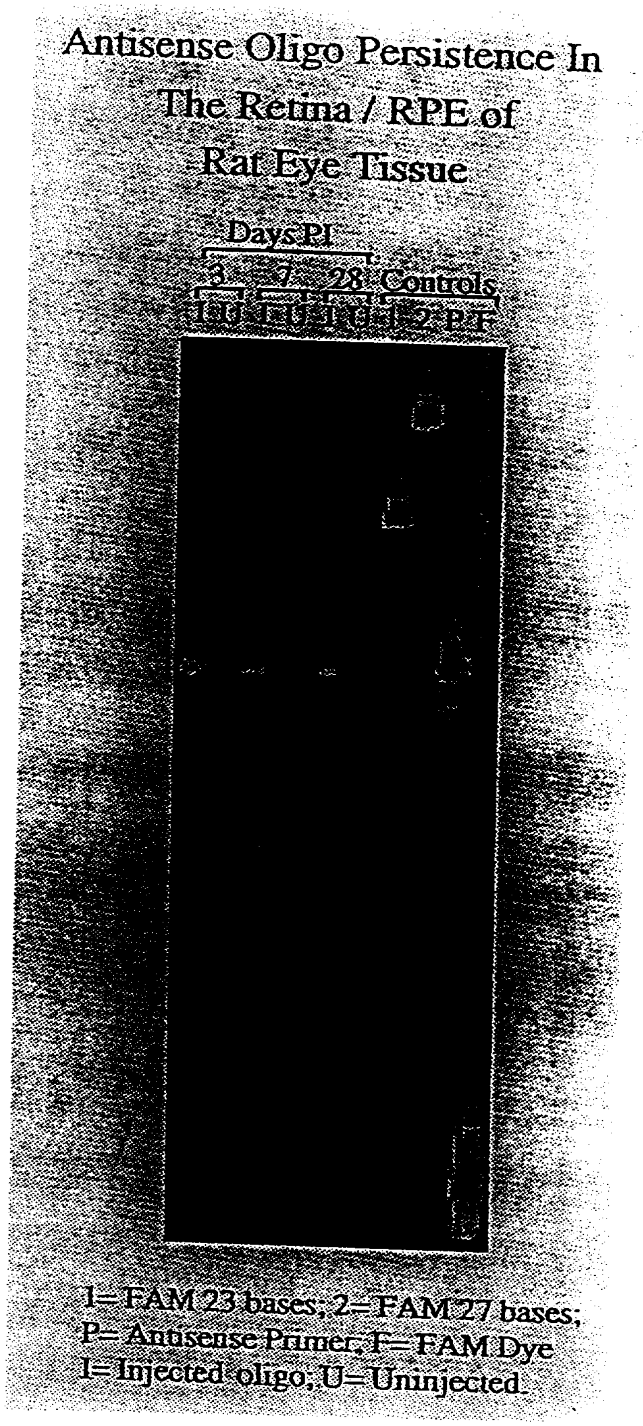

Antisense Oligo Persistence In
The Retina / RPE of
Rat Eye Tissue
Days PI
3 7 28 Controls
I U I U I U 1 2 P F
1= FAM 23 bases; 2= FAM 27 bases;
P= Antisense Primer; F= FAM Dye
I= Injected oligo; U= Uninjected

FIGURE 1a

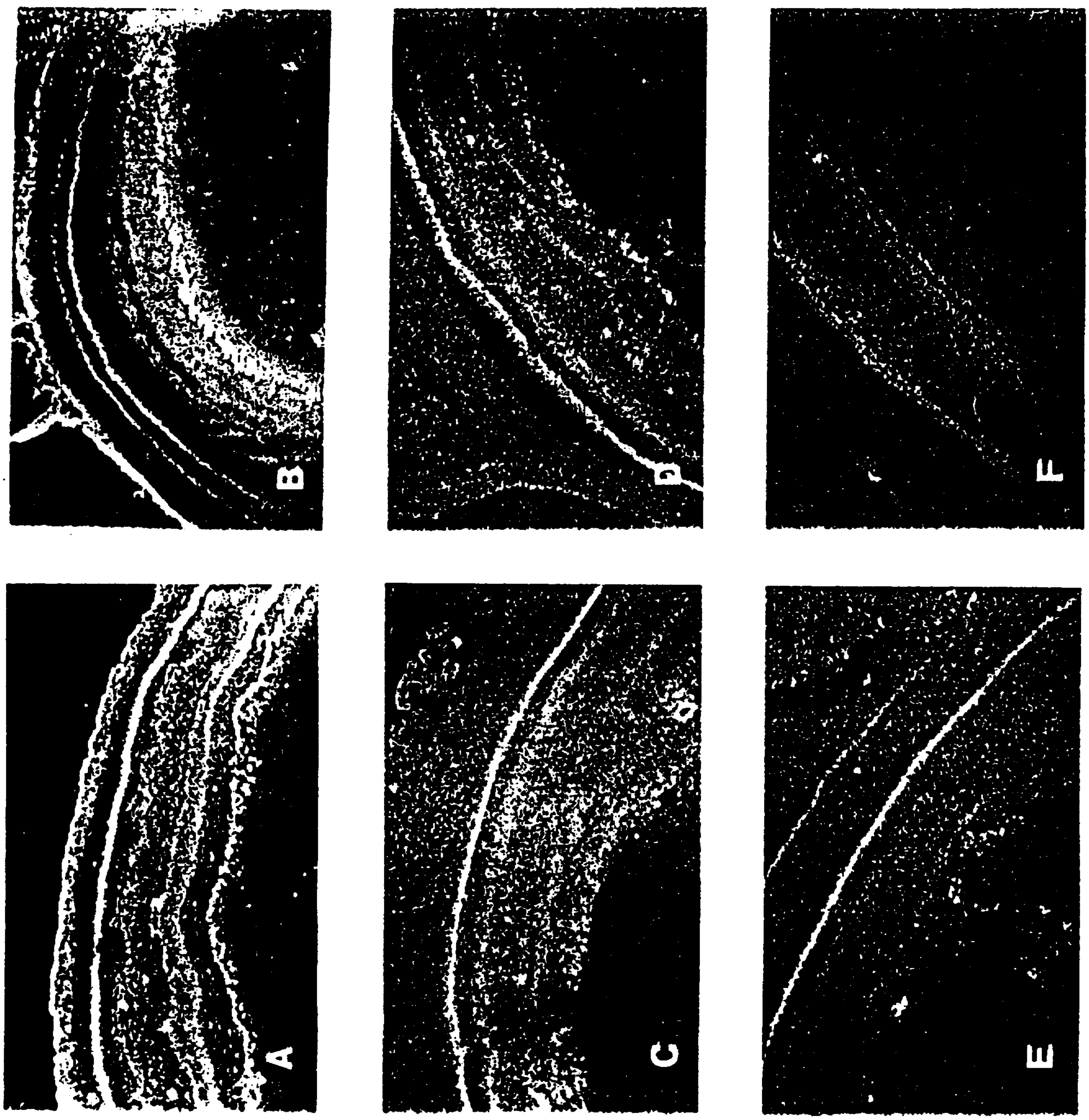
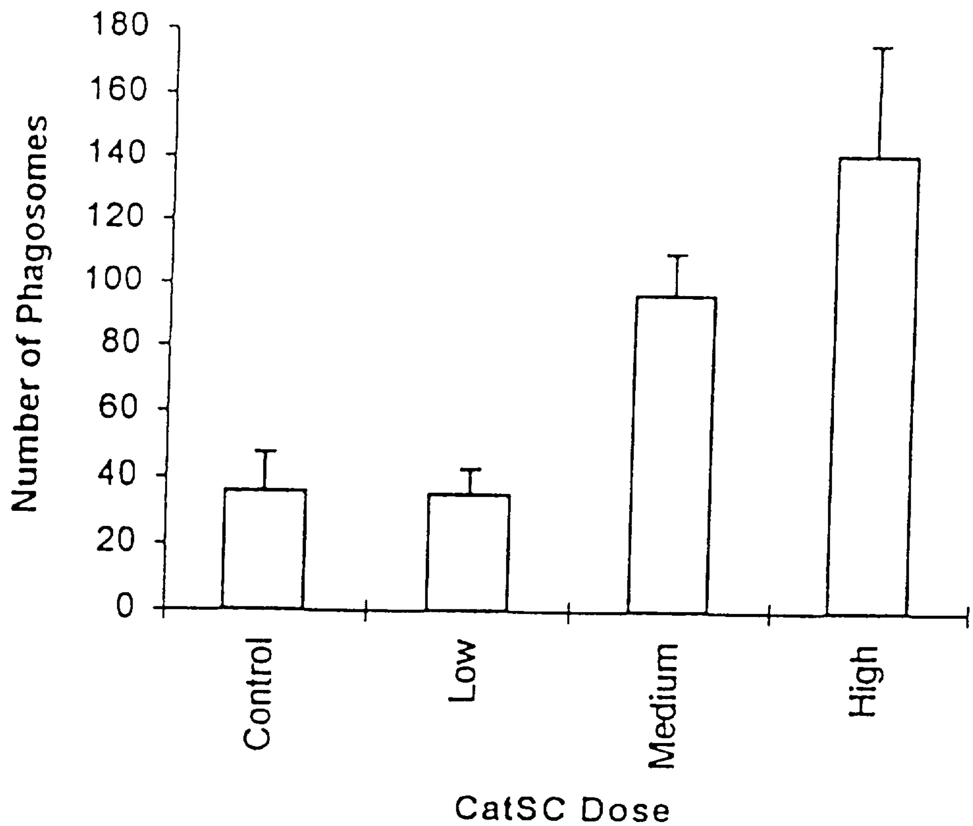


FIGURE 1b



180
160
140
120
100
80
60
40
20
0
Number of Phagosomes
Control
Low
Medium
High
CatSC Dose

FIGURE 2

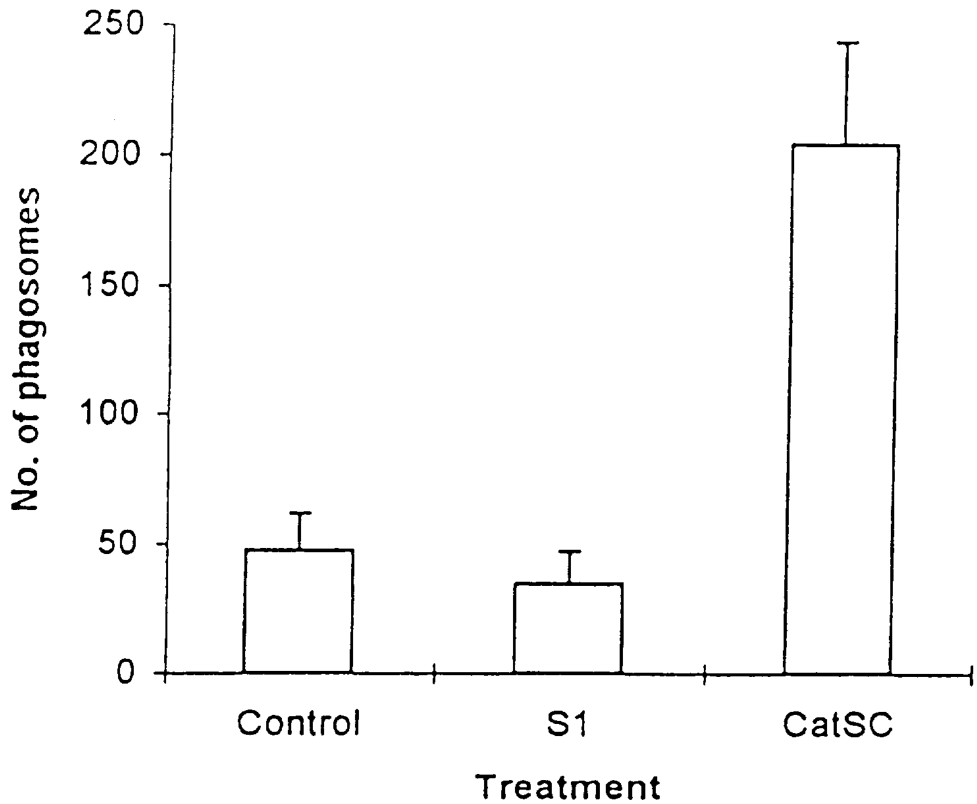

250
200
150
100
50
0
No. of phagosomes
Control
S1
CatSC
Treatment

FIGURE 3

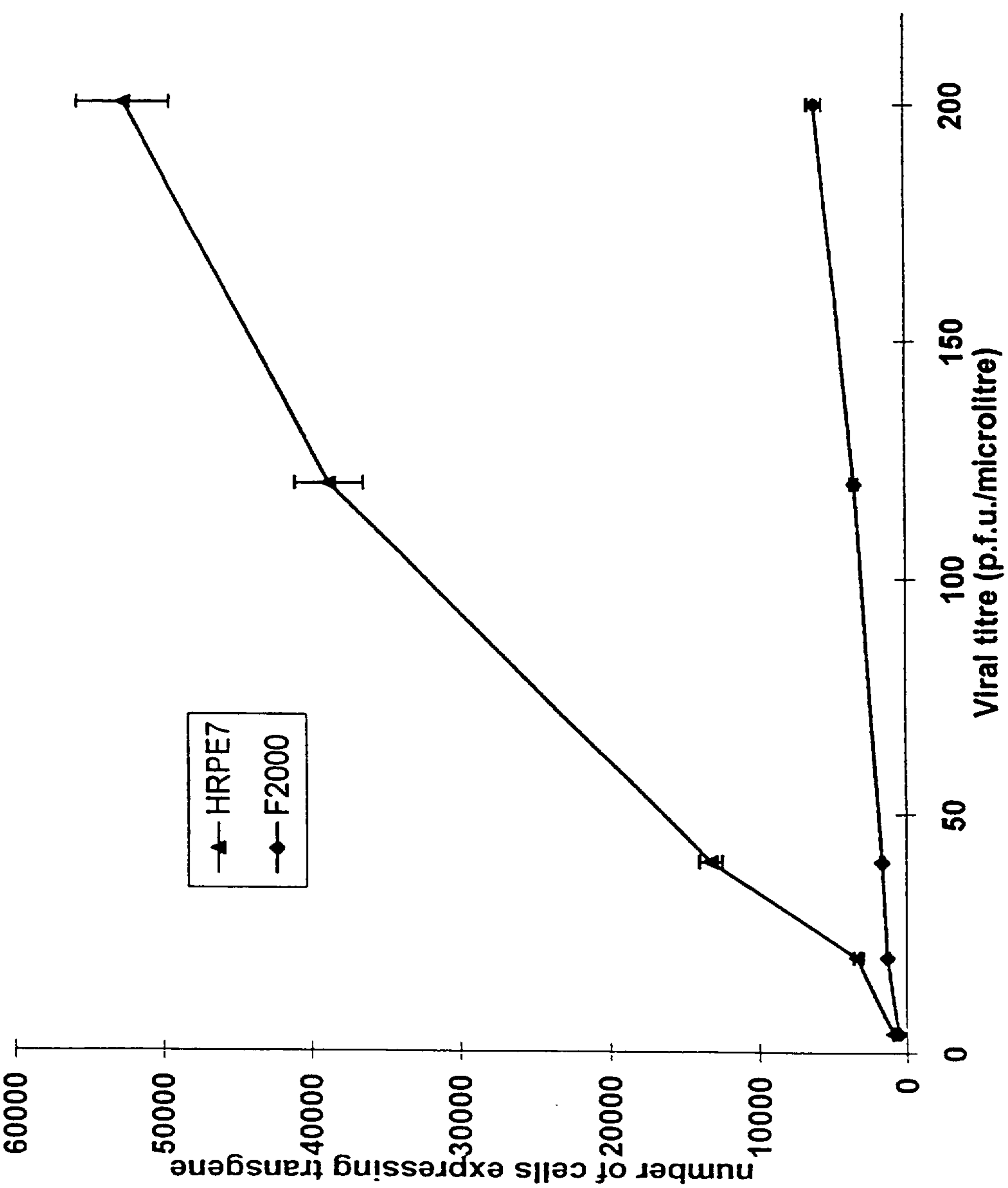

HRPE7
F2000
number of cells expressing transgene
60000
50000
40000
30000
20000
10000
0
0
50
100
150
200
Viral titre (p.f.u./microlitre)

FIGURE 4

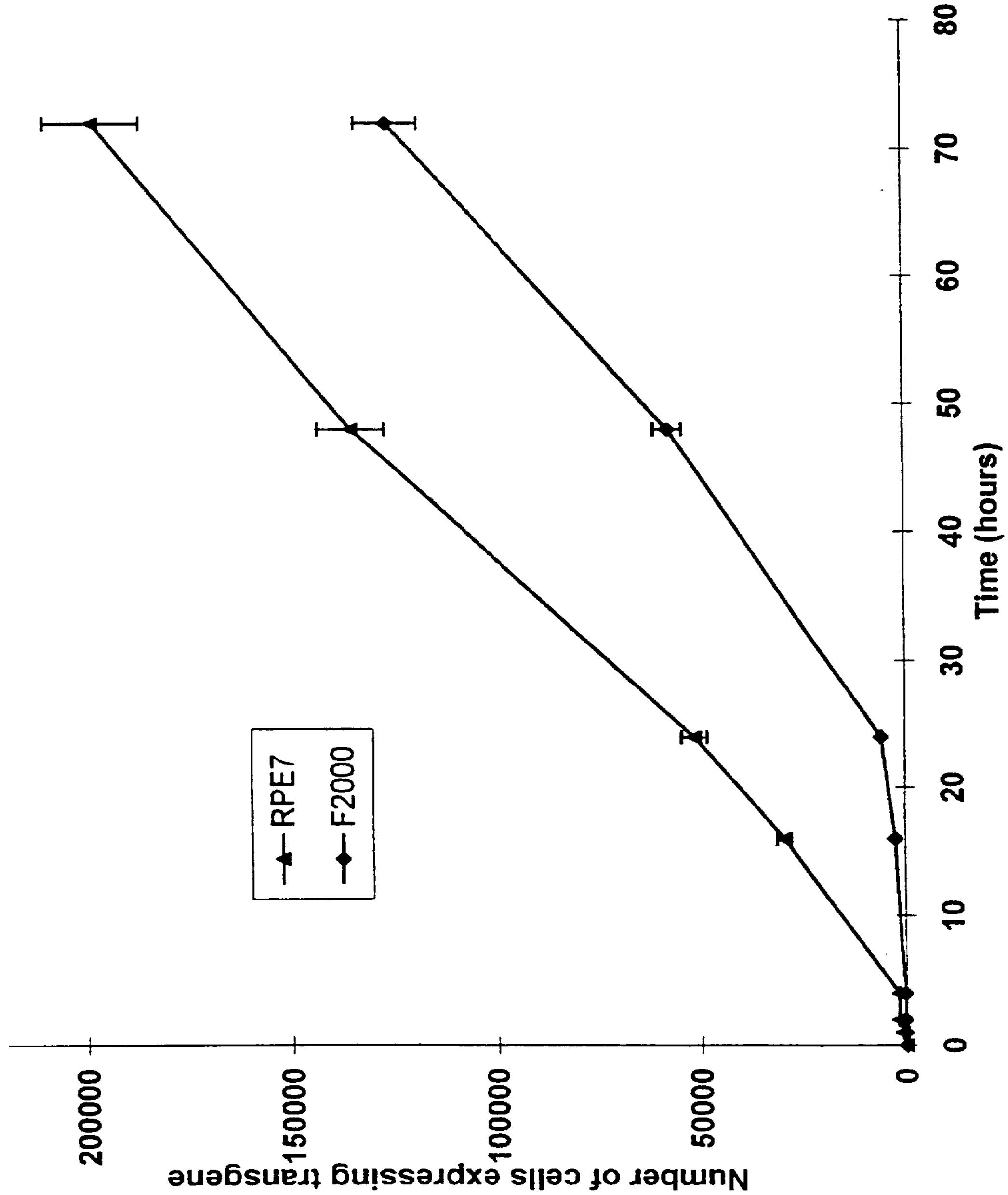

RPE7
F2000
Number of cells expressing transgene
200000
150000
100000
50000
0
Time (hours)
0
10
20
30
40
50
60
70
80

FIGURE 5

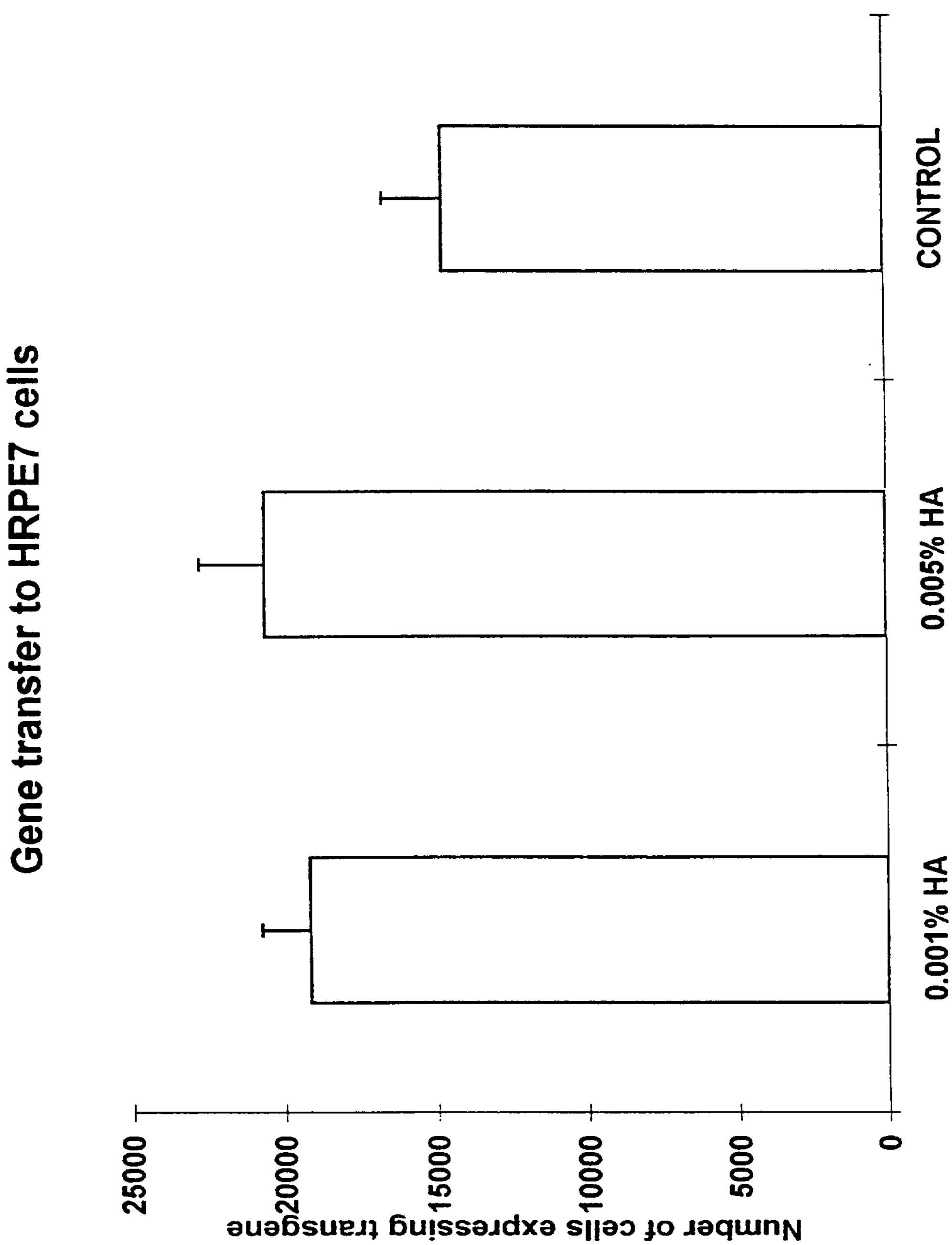

Gene transfer to HRPE7 cells
Number of cells expressing transgene
25000
20000
15000
10000
5000
0
0.001% HA
0.005% HA
CONTROL

FIGURE 6

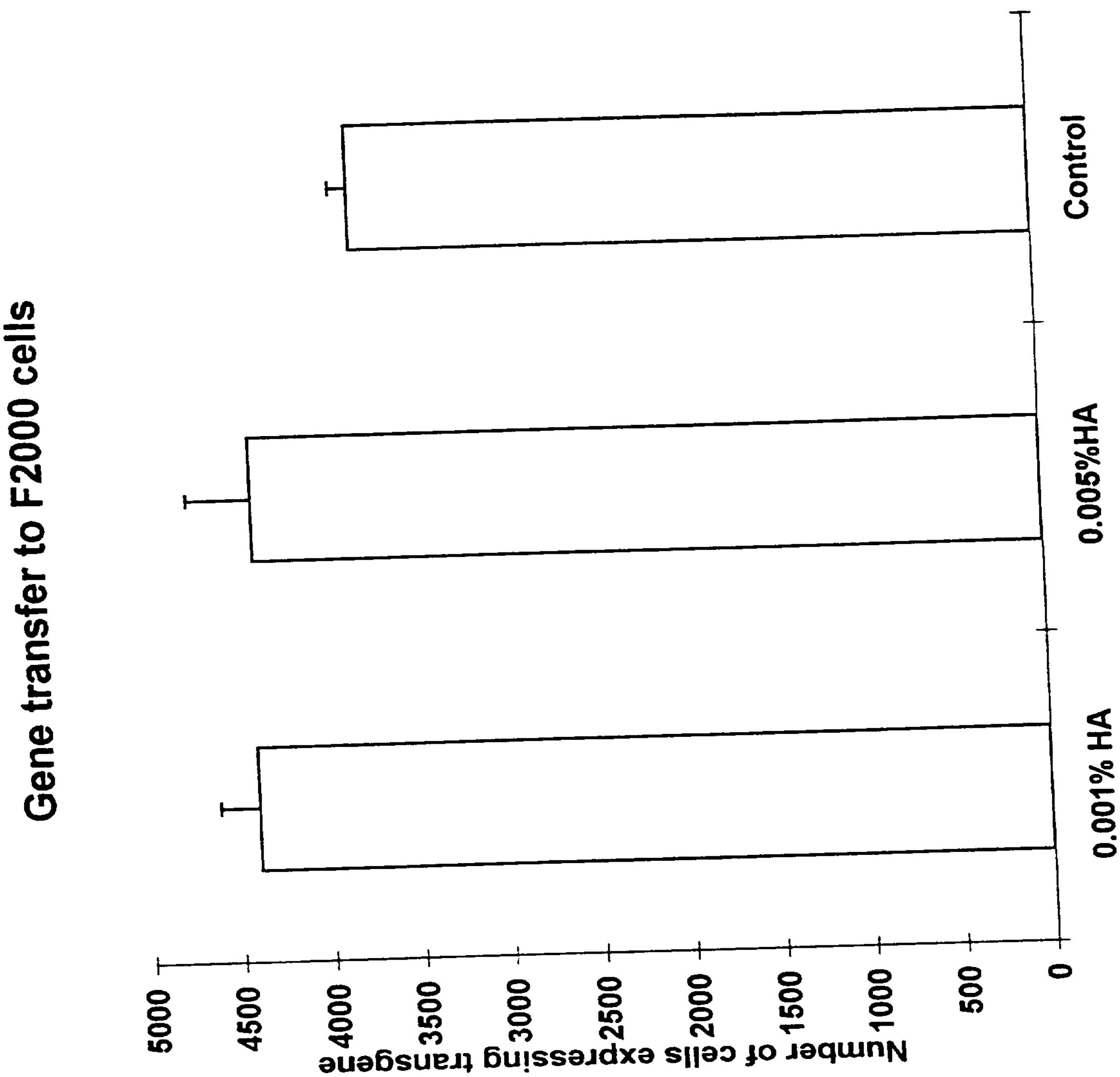

Gene transfer to F2000 cells
Number of cells expressing transgene
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0.001% HA
0.005%HA
Control

FIGURE 7

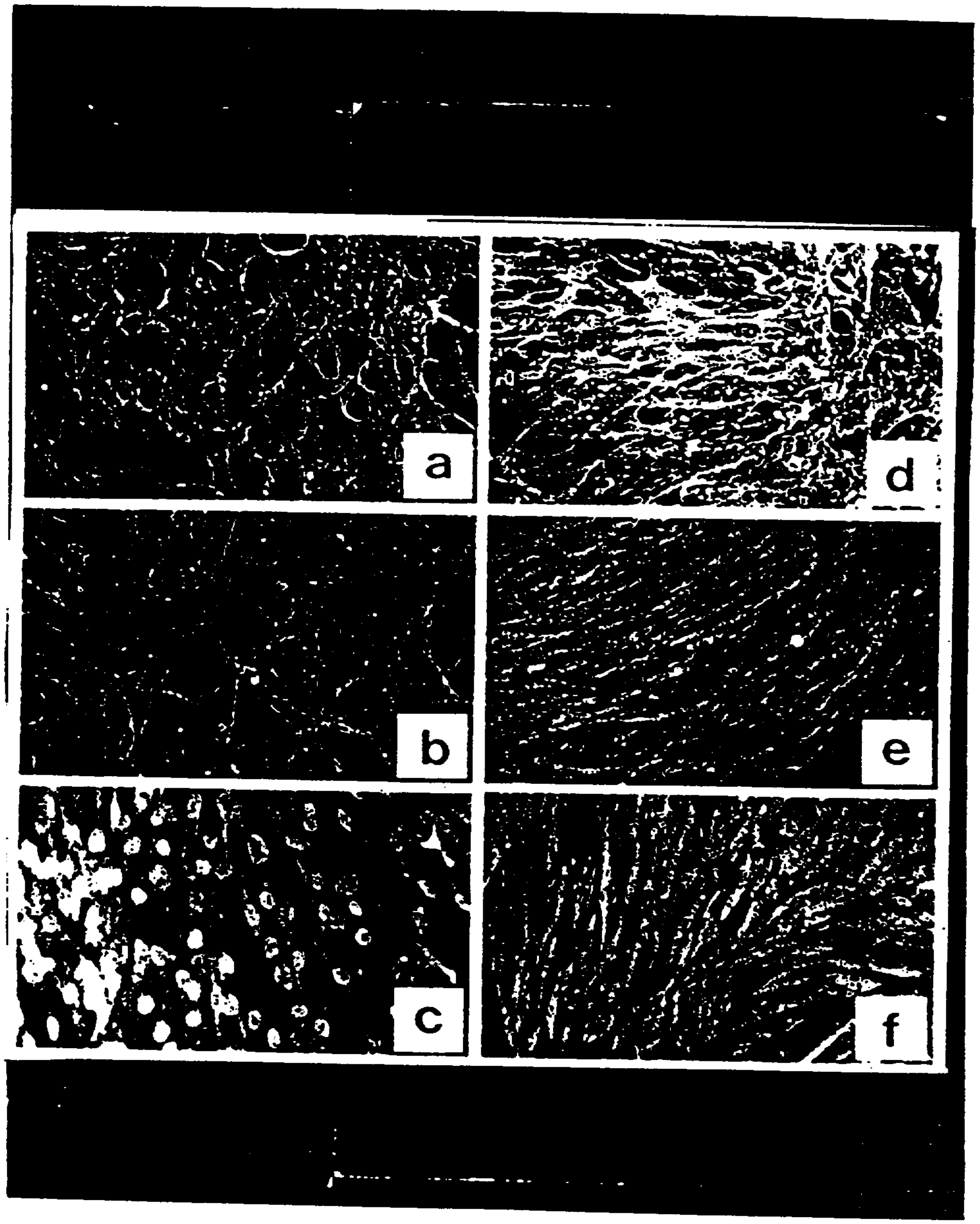


FIGURE 8

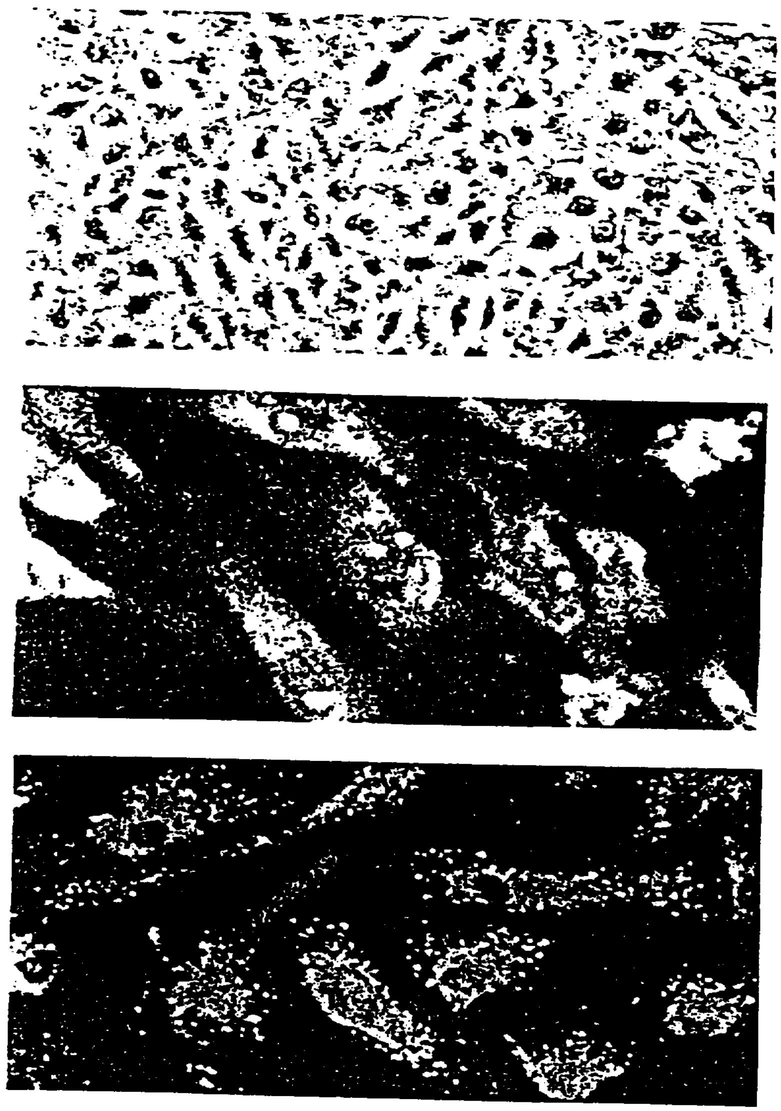


FIGURE 9

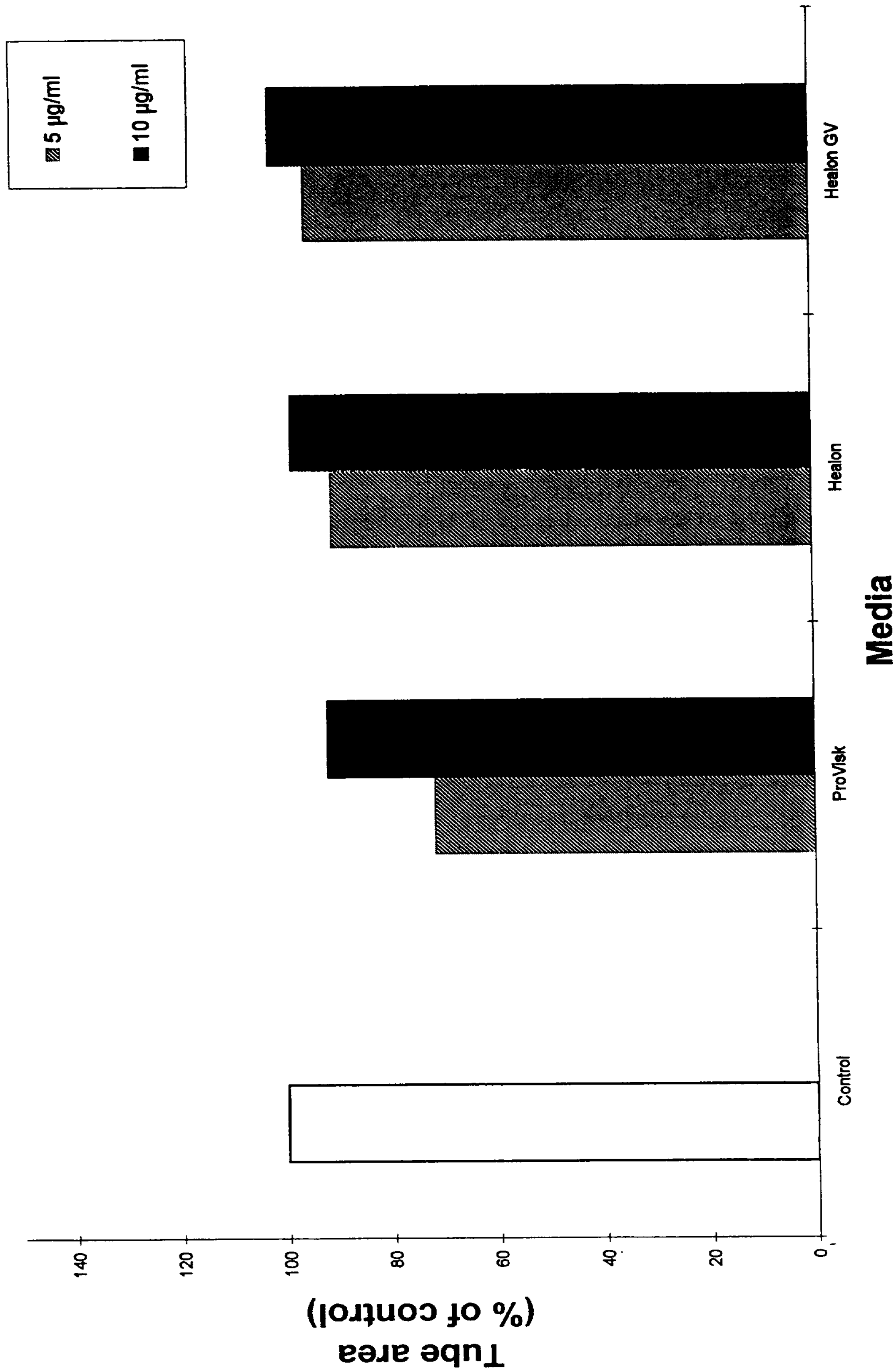

5 µg/ml
10 µg/ml
Healon GV
Healon
ProVisk
Control
Media
0
20
40
60
80
100
120
140
Tube area
(% of control)

FIGURE 10

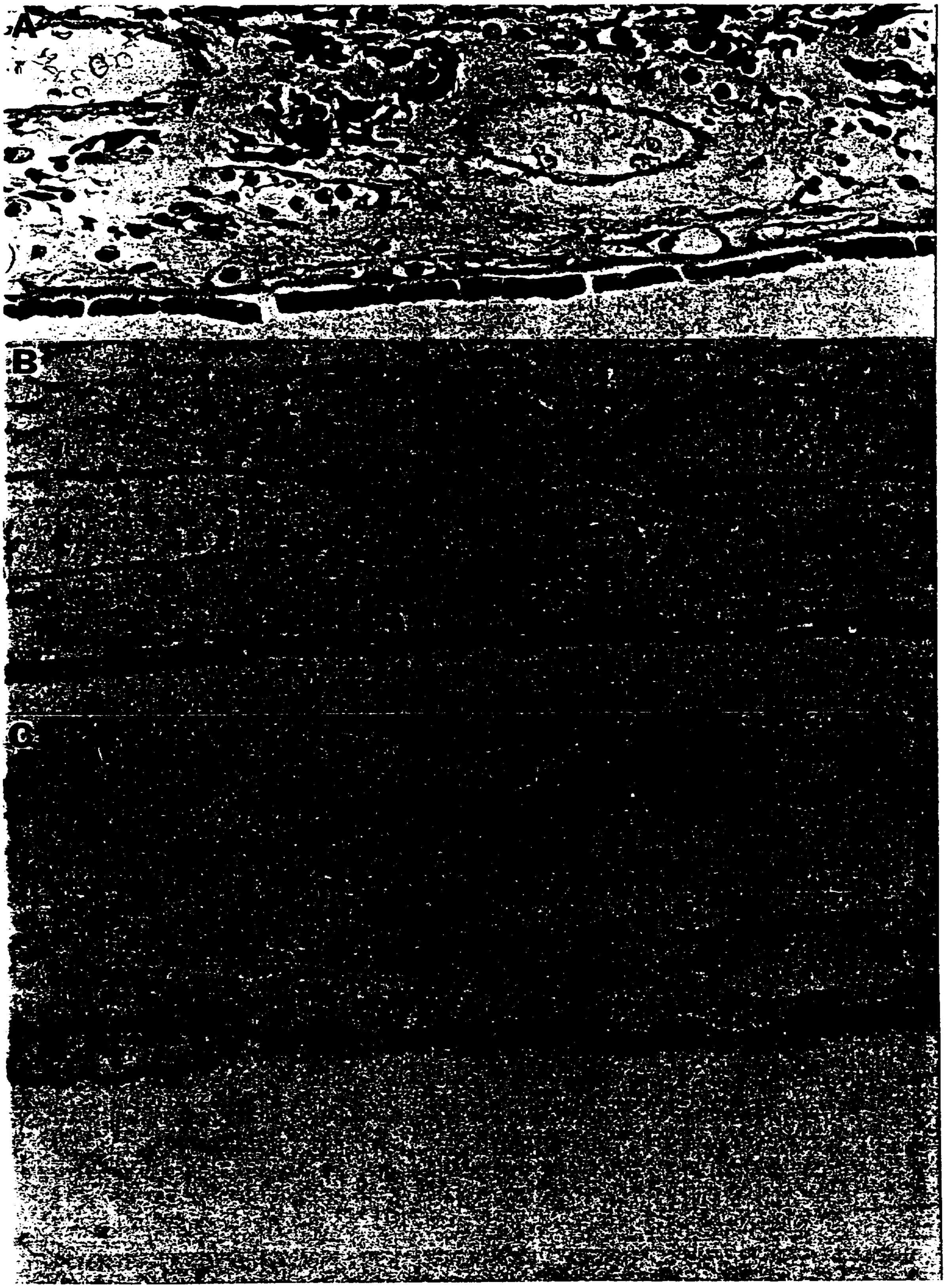


FIGURE 11

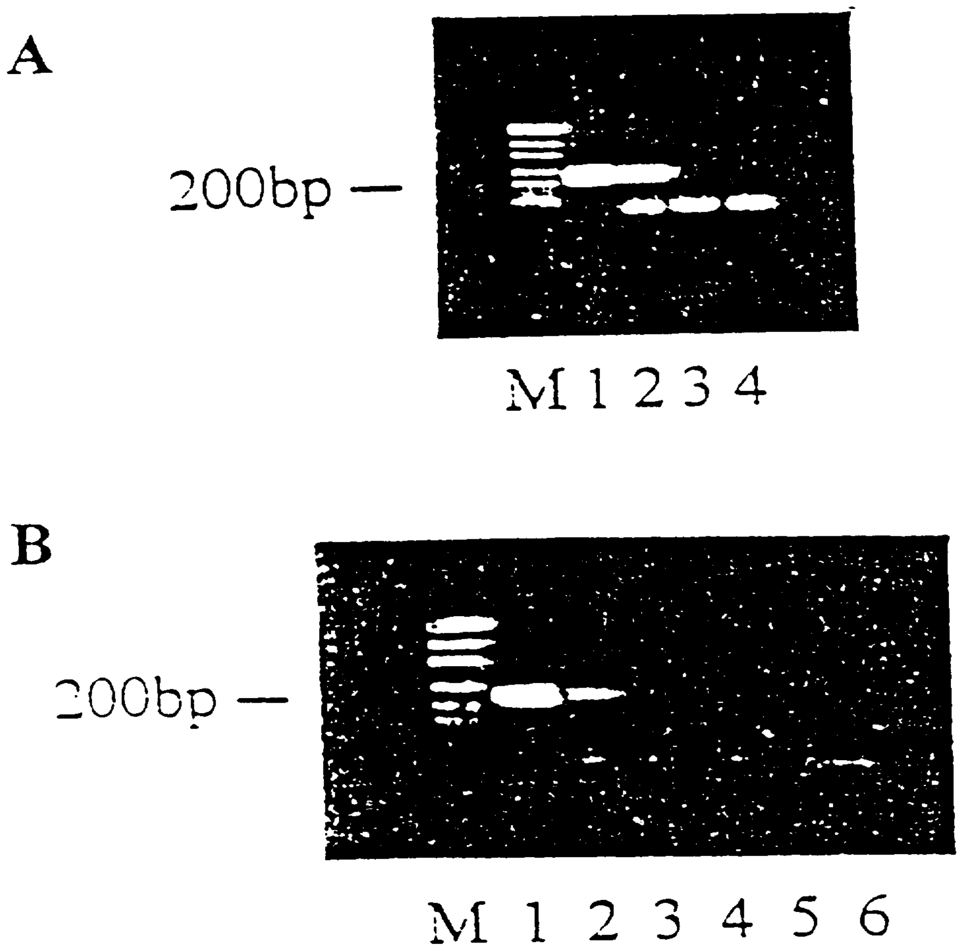


FIGURE 12

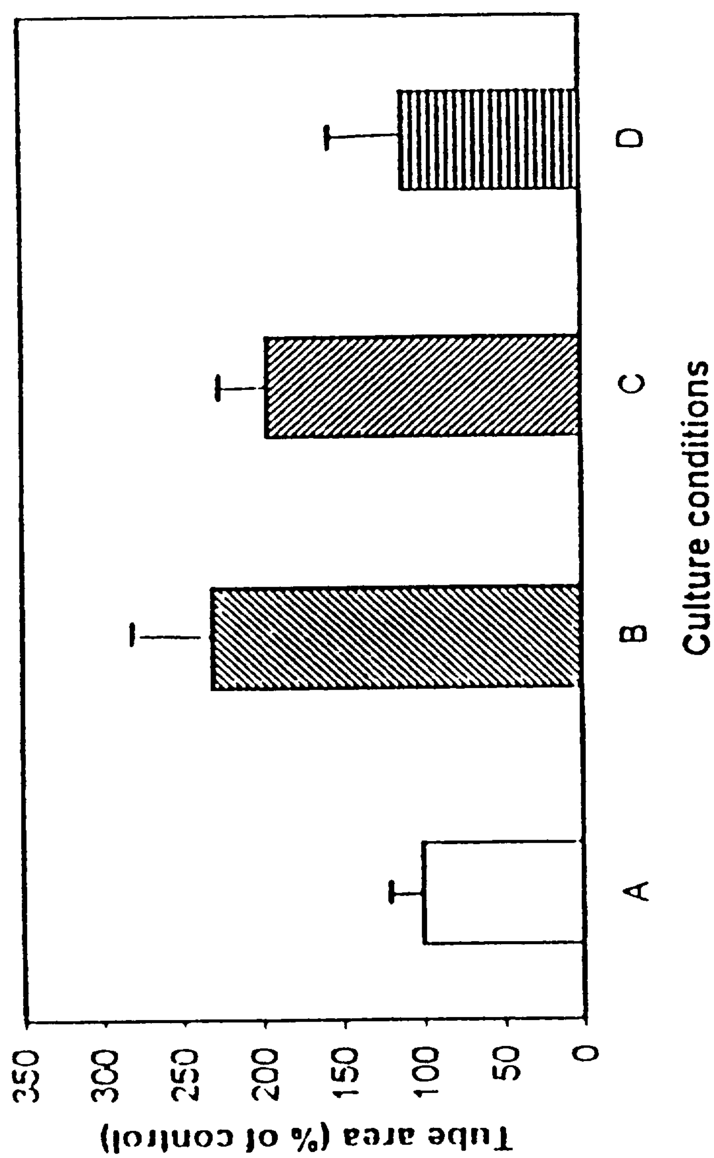

Tube area (% of control)
0
50
100
150
200
250
300
350
A
B
C
D
Culture conditions

FIGURE 13

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00664

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61K 47/36 A61K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Key words as for {B49}

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU IPC A61K 47/36; 48/00

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT, JAPIO, CA, BIOTEC, MEDLINE
Nucleic()acid and hyaluronic()acid
VEGF and antisense

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO-A-90/02774 (PIER AUGE) 22 March 1990 Claim 1	1
Y	AU-A-60903/86 (BIOMATRIX) 5 August 1986 Particularly pages 4-5	1-31, 55-58
Y	AU-A-52274/93 (NORMPHARM CO. INC) 3 March 1994 Particularly pages 17-19	1-31, 55-58

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
6 January 1997

Date of mailing of the international search report
31 JAN 1997

Name and mailing address of the ISA/AU
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION
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WODEN ACT 2606
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C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	WO-A-96/23065 (Hybridon Inc) 1 August 1996 Whole document	32-54
X Y	WO-A-95/04142 (Hybridon Inc) 9 February 1995 Whole document	32-54 22-31
X, P	WO-A-96/00286 (TOAGOSEI Co Ltd) 4 January 1996 Page 35	32-54
X, P	WO-A-96/27006 (Hybridon Inc) 6 September 1996 Whole document	32-54
Y	WO-A-92/13063 (Oncogene Science) 6 August 1992 Page 31, lines 17-30	32-54
Y	Uhlman and Peyman "Antisense Oligo nucleotides" Chemical Reviews 1990 Vol. 90 No. 4 pages 544-579	22-31, 32-54

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00664

Box 1 **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Claims 1-31 and 55-58 are to compositions and treatments comprising hyaluronic acid and nucleic acids. Claims 32-54 are to compositions and treatments comprising nucleic acids without hyaluronic acid as an essential component.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 96/00664

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	90/02774	EP	386216	AU	42171/89	US	5194253
AU	60903/86	EP	224987	US	5128326		
AU	52274/93	EP	445255	EP	656213	WO	91/04058
WO	96/23065	AU	49074/96				
WO	95/04142	EP	716688				
WO	96/27006	AU	51791/96				
WO	96/00286	JP	8070899				
WO	92/13063	AU	14692/92				

END OF ANNEX